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[54] **STABILIZED MICROBUBBLE COMPOSITIONS**

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[58] **Field of Search** 424/9.5, 9.51, 424/9.52

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[57] **ABSTRACT**

A microbubble preparation formed of a plurality of microbubbles comprising a first gas and a second gas surrounded by a membrane such as a surfactant, wherein the first gas and the second gas are present in a molar ratio of from about 1:100 to about 1000:1, and wherein the first gas has a vapor pressure of at least about (760–x) mm Hg at 37° C., where x is the vapor pressure of the second gas at 37° C., and wherein the vapor pressure of each of the first and second gases is greater than about 75 mm Hg at 37° C.; also disclosed are methods for preparing microbubble compositions, including compositions that rapidly shrink from a first average diameter to a second average diameter less than about 75% of the first average diameter and are stabilized at the second average diameter; kits for preparing microbubbles; and methods for using such microbubbles as ultrasound contrast agents.

52 Claims, No Drawings

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STABILIZED MICROBUBBLE COMPOSITIONS

This application is a continuation of application Ser. No. 08/099,951, filed Jul. 30, 1993, now abandoned.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention includes a method for preparing stable long-lived microbubbles for ultrasound contrast enhancement and other uses, and to compositions of the bubbles so prepared.

2. Background of the Art

Ultrasound technology provides an important and more economical alternative to imaging techniques which use ionizing radiation. While numerous conventional imaging technologies are available, e.g., magnetic resonance imaging (MRI), computerized tomography (CT), and positron emission tomography (PET), each of these techniques use extremely expensive equipment. Moreover, CT and PET utilize ionizing radiation. Unlike these techniques, ultrasound imaging equipment is relatively inexpensive. Moreover, ultrasound imaging does not use ionizing radiation.

Ultrasound imaging makes use of differences in tissue density and composition that affect the reflection of sound waves by those tissues. Images are especially sharp where there are distinct variations in tissue density or compressibility, such as at tissue interfaces. Interfaces between solid tissues, the skeletal system, and various organs and/or tumors are readily imaged with ultrasound.

Accordingly, in many imaging applications ultrasound performs suitably without use of contrast enhancement agents; however, for other applications, such as visualization of flowing blood in tissues, there have been ongoing efforts to develop such agents to provide contrast enhancement. One particularly significant application for such contrast agents is in the area of vascular imaging. Such ultrasound contrast agents could improve imaging of flowing blood in the heart, kidneys, lungs, and other tissues. This, in turn, would facilitate research, diagnosis, surgery, and therapy related to the imaged tissues. A blood pool contrast agent would also allow imaging on the basis of blood content (e.g., tumors and inflamed tissues) and would aid in the visualization of the placenta and fetus by enhancing only the maternal circulation.

A variety of ultrasound contrast enhancement agents have been proposed. The most successful agents have generally consisted of microbubbles that can be injected intravenously. In their simplest embodiment, microbubbles are miniature bubbles containing a gas, such as air, and are formed through the use of foaming agents, surfactants, or encapsulating agents. The microbubbles then provide a physical object in the flowing blood that is of a different density and a much higher compressibility than the surrounding fluid tissue and blood. As a result, these microbubbles can easily be imaged with ultrasound.

Most microbubble compositions have failed, however, to provide contrast enhancement that lasts even a few seconds, let alone minutes, of contrast enhancement. This greatly limits their usefulness. Microbubbles have therefore been "constructed" in various manners in an attempt to increase their effective contrast enhancement life. Various avenues have been pursued: use of different surfactants or foaming agents; use of gelatins or albumin microspheres that are initially formed in liquid suspension, and which entrap gas during solidification; and liposome formation. Each of these attempts, in theory, should act to create stronger bubble structures. However, the entrapped gases (typically air, CO_2, and the like) are under increased pressure in the bubble due to the surface tension of the surrounding surfactant, as described by the La Place equation ($\Delta P=2\gamma/r$).

This increased pressure, in turn, results in rapid shrinkage and disappearance of the bubble as the gas moves from a high pressure area (in the bubble) to a lower pressure environment (in either the surrounding liquid which is not saturated with gas at this elevated pressure, or into a larger diameter, lower pressure bubble).

Solid phase shells that encapsulate gases have generally proven too fragile or to permeable to the gas to have satisfactory in vivo life. Furthermore, thick shells (e.g., albumin, sugar, or other viscous materials) reduce the compressibility of the bubbles, thereby reducing their echogenicity during the short time they can exist. Solid particles or liquid emulsion droplets that evolve gas or boil when injected pose the danger of supersaturating the blood with the gas or vapor. This will lead to a small number of large embolizing bubbles forming at the few available nucleation sites rather than the intended large number of small bubbles.

One proposal for dealing with such problems is outlined in Quay, PCT/US92/07250. Quay forms bubbles using gases selected on the basis of being a gas at body temperature (below 37° C.), and having reduced water solubility, higher density, and reduced gas diffusivity in solution in comparison to air. Although reduced water solubility and diffusivity can affect the rate at which the gas leaves the bubble, numerous problems remain with the Quay bubbles. Forming bubbles of sufficiently small diameter (e.g., 0.2 μm) requires high energy input. This is a disadvantage in that sophisticated bubble preparation systems must be provided at the site of use. Moreover, The Quay gas selection criteria are incorrect in that they fail to consider certain major causes of bubble shrinkage, namely, the effects of bubble surface tension, surfactants and gas osmotic effects, and these errors result in the inclusion of certain unsuitable gases and the exclusion of certain optimally suitable gases.

Accordingly, a need exists in the art for compositions, and a method to prepare such compositions, that provide, or utilize, a longer life contrast enhancement agent that is biocompatible, easily prepared, and provides superior contrast enhancement in ultrasound imaging.

SUMMARY OF THE INVENTION

In accordance with the present invention, there is provided an ultrasound contrast enhancement agent that has a prolonged longevity in vivo, which consists of virtually any conventional microbubble formulation in conjunction with an entrapped gas or gas mixture that is selected based upon consideration of partial pressures of gases both inside and outside of the bubble, and on the resulting differences in gas osmotic pressure that oppose bubble shrinkage. Gases having a low vapor pressure and limited solubility in blood or serum (i.e., relatively hydrophobic) may advantageously be provided in combination with another gas that is more rapidly exchanged with gases present in normal blood or serum. Surfactant families allowing the use of higher molecular weight gas osmotic agents, and improved methods of bubble production are also disclosed.

One aspect of the present invention is a stabilized gas filled microbubble preparation, comprising a mixture of a first gas or gases and a second gas or gases within generally

spherical membranes to form microbubbles, wherein the first gas and the second gas are respectively present in a molar ratio of about 1:100 to about 1000:1, and wherein the first gas has a vapor pressure of at least about (760–x) mm Hg at 37° C., where x is the vapor pressure of the second gas at 37° C., and wherein the vapor pressure of each of the first and second gases is greater than about 75 mm Hg at 37° C., with the proviso that the first gas and the second gas are not water vapor. In one embodiment, the second gas comprises a fluorocarbon and the first gas is a nonfluorocarbon, such as nitrogen, oxygen, carbon dioxide, or a mixture thereof.

The microbubbles may advantageously be provided in a liquid medium, such as an aqueous medium, wherein they have a first average diameter, the ratio of the first gas to the second gas in the microbubbles is at least 1:1, and the microbubbles are adapted to shrink in the medium as a result of loss of the first gas through the membrane to a second average diameter of less than about 75% of the first diameter and then remain stabilized at or about the second diameter for at least about 1 minute as a result of a gas osmotic pressure differential across the membrane. Advantageously, the medium is in a container and the microbubbles have actually been formed in the container. Alternatively, the medium is blood in vivo. In one embodiment, the liquid medium contains gas or gases dissolved therein with a gas tension of at least about 700 mm Hg, wherein the first diameter is at least about 5 μm, and wherein the tension of the gas or gases dissolved in the medium is less than the partial pressure of the same gas or gases inside the microbubbles.

In a particularly preferred embodiment, the bubble initially contains at least three gases: a first gas having a partial pressure far greater than the gas tension of the same gas in the surrounding liquid (e.g., 1.5, 2, 4, 5, 10, 20, 50, or 100 or more times greater than in the surrounding liquid); a second gas that is retained in the bubble due to a relatively low permeability of the bubble membrane to the gas, or a relatively low solubility of the gas in the surrounding medium (as described elsewhere herein), and a third gas to which the membrane is relatively permeable that is also present in the surrounding medium. For example, in an aqueous system exposed to or at least partially equilibrated with air (such as blood), the first gas may advantageously be carbon dioxide or another gas not present in large quantities in air or blood; the second gas may be a fluorocarbon gas, such as perfluorohexane; and the third gas may be air or a major component of air such as nitrogen or oxygen.

Preferably, the first diameter prior to shrinkage is at least about 10 μm and the second diameter at which the diameter is stabilized is between about 1 μm and 6 μm.

For all of the microbubble preparations or methods described herein, in one preferred embodiment, the second gas has an average molecular weight at least about 4 times that of the first gas. In another preferred embodiment, the second gas has a vapor pressure less than about 750 or 760 mm Hg at 37° C. Moreover, it is preferred that the molar ratio of the first gas to the second gas is from about 1:10 to about 500:1, 200:1, or 100:1. In other preferred embodiments, the second gas comprises a fluorocarbon or a mixture of at least two or three fluorocarbons, and the first gas is a nonfluorocarbon. In some advantageous preparations, the second gas comprises one or more perfluorocarbons. In others, both the first gas and the second gas comprise fluorocarbons. In still others, the microbubbles contain as the first gas, or as the second gas, or respectively as the first and second gases, gaseous perfluorobutane and perfluorohexane in a ratio from about 1:10 to about 10:1. Alternatively, the microbubbles contain as the first gas, or as the second gas, or respectively as the first and second gases, gaseous perfluorobutane and perfluoropentane in a ratio from about 1:10 to about 10:1. It is advantageous that the second gas leave the microbubble much more slowly than does the first gas; thus, it is preferred that the second gas has a water solubility of not more than about 0.5 mM at 25° C. and one atmosphere, and the first gas has a water solubility at least about 10 times, and preferably at least 20, 50, 100, or 200 times greater than that of the second gas. Similarly, it is preferred that the permeability of the membrane to the first gas is at least about 5 times, preferably 10, 20, 50, or 100 times greater than the permeability of the membrane to the second gas.

The microbubble preparation may advantageously be contained in a container, having a liquid in the container in admixture with the microbubbles, wherein the container further comprises means for transmission of sufficient ultrasonic energy to the liquid to permit formation of the microbubbles by sonication. In this way, the microbubbles can be formed by the physician (or other professional) immediately before use by applying ultrasonic energy from an outside source to the sterile preparation inside the container. This means for transmission can, for example, be a flexible polymer material having a thickness less than about 0.5 mm (which permits ready transmission of ultrasonic energy without overheating the membrane). Such membranes can be prepared from such polymers as natural or synthetic rubber or other elastomer, polytetrafluoroethylene, polyethylene terephthalate, and the like.

In the microbubble preparations of the invention, the membrane enclosing the gas is preferably a surfactant. One preferred type of surfactant comprises a non-Newtonian viscoelastic surfactant, alone or in combination with another surfactant. Other preferred general and specific categories of surfactants include carbohydrates, such as polysaccharides, derivatized carbohydrates, such as fatty acid esters of sugars such as sucrose (preferably sucrose stearate), and proteinaceous surfactants including albumin. Alternatively, the membrane of the microbubble need not be a fluid (such as a surfactant), but instead can be a solid or semi-solid, such as hardened, thickened, or denatured proteinaceous material (e.g. albumin), carbohydrates, and the like.

One advantageous form of the invention is a kit for use in preparing microbubbles, preferably at the site of use. This kit may comprise sealed container (such as a vial with a septum seal for easy removal of the microbubbles using a hypodermic syringe), a liquid in the container (such as water or a buffered, isotonic, sterile aqueous medium), a surfactant in the container, and a fluorocarbon gas (including a fluorocarbon vapor) in the container, wherein the liquid, the surfactant, and the fluorocarbon gas or vapor are together adapted to form microbubbles upon the application of energy thereto. The energy advantageously may be simple shaking energy, either manual or mechanical, stirring or whipping, or ultrasonic energy. The kit preferably includes means in the container for permitting transmission of sufficient external ultrasonic energy to the liquid to form microbubbles in the container. As above, the means for transmission can in one embodiment comprise a flexible polymer membrane having a thickness less than about 0.5 mm. In one embodiment, the kit further includes a nonfluorocarbon gas in the container, wherein the molar ratio of the nonfluorocarbon gas to the fluorocarbon gas is from about 1:10 to about 1000:1, with the proviso that the nonfluorocarbon gas is not water vapor. In all of the kits of the present invention, the surfactant, the gas or gases, and the other

vapor pressure of the gas osmotic agent is at least 100 Torr at 37° C. For in vivo imaging mean bubble diameters between 1 and 10 μm are preferred, with 3 to 5 μm most preferred. The invention may in one embodiment also be described as a mixture of a first gas or gases and a second gas or gases within generally spherical membranes to form microbubbles, where the first gas and the second gas are respectively present in a molar ratio of about 1:100, 1:75, 1:50, 1:30, 1:20, or 1:10 to about 1000:1, 500:1, 250:1, 100:1, 75:1 or 50:1, and where the first gas has a vapor pressure of at least about (760–x) mm Hg at 37° C., where x is the vapor pressure of the second gas at 37° C., and where the vapor pressure of each of the first and second gases is greater than about 75 or 100 mm Hg at 37° C.

Microbubbles prepared in accordance with one preferred embodiment of the invention may also possess an additional advantageous property. In one such embodiment, mixtures of nonosmotic gases with osmotic stabilizing gases (or gas osmotic agents) are used to stabilize the resultant bubble size distribution during and immediately after production. Upon generation of the bubbles, the higher LaPlace pressure in smaller bubbles causes diffusion through the liquid phase to the lower La Place pressure larger bubbles. This causes the mean size distribution to increase above the capillary dimension limit of 5 microns over time. This is called disproportionation. When a mixture of a nonosmotic gas (e.g., air) is used with an osmotic vapor (e.g., C_6F_{14}) a slight reduction in volume of the smaller bubbles, due to air leaving the bubble, concentrates the osmotic gas and increases its osmotic pressure thus retarding further shrinkage while the larger bubbles increase in volume slightly, diluting the osmotic gas and retarding further growth.

An additional advantage of using a mixture of an extremely blood soluble gases (e.g., 87.5% by volume CO_2) and an osmotic gas mixture (e.g., 28% C_6F_{14} vapor+72% air) is that, when injected, these bubbles rapidly shrink due to the loss of CO_2 to the blood. The bubbles, upon injection, will experience an 87.5% volume decrease due to loss of CO_2. This loss of CO_2 corresponds to a halving of the bubble diameter. Accordingly, one can prepare larger diameter bubbles (e.g., 9 μm), using simplified mechanical means, that will shrink to below 5 microns upon injection. In general, such bubbles will initially be prepared where the first gas is present in a ratio of at least 1:1 with respect to the second gas, preferably at least 3:2, 2:1, 3:1, 4:1, 5:1, or 10:1. Where the microbubble membrane is more permeable to the first gas than to the second gas (e.g., the membrane has respective permeabilities to the gases in a ratio of at least about 2:1, 3:1, 4:1, 5:1, or 10:1, preferably even higher, e.g., 20:1, 40:1, or 100:1), the bubbles advantageously shrink from their original first diameter to an average second diameter of 75% or less of their original diameter quite rapidly (e.g., within one, two, four, or five minutes). Then, when at least one relatively membrane-permeable gas is present in the aqueous medium surrounding the microbubble, the bubble is preferably stabilized at or about the second diameter for at least about 1 minute, preferably for 2, 3, 4, or 5 minutes. In one preferred embodiment, the bubbles maintain a size between about 5 or 6 μm and 1 μm for at least 1, 2, 3, 4, or 5 minutes, stabilized by a gas osmotic pressure differential. The gas tension in the external liquid is preferably at least about 700 mm Hg. Moreover, a relatively membrane impermeable gas is also in the microbubble to create such an osmotic pressure differential.

I. Microbubble Construction

A. The Aqueous or Other Liquid Phase

The external, continuous liquid phase in which the bubble resides typically includes a surfactant or foaming agent. Surfactants suitable for use in the present invention include any compound or composition that aids in the formation and maintenance of the bubble membrane by forming a layer at the interface between the phases. The foaming agent or surfactant may comprise a single compound or any combination of compounds, such as in the case of co-surfactants.

Examples of suitable surfactants or foaming agents include: block copolymers of polyoxypropylene polyoxyethylene, sugar esters, fatty alcohols, aliphatic amine oxides, hyaluronic acid aliphatic esters, hyaluronic acid aliphatic ester salts, dodecyl poly(ethyleneoxy)ethanol, nonylphenoxy poly(ethyleneoxy)ethanol, hydroxy ethyl starch, hydroxy ethyl starch fatty acid esters, dextrans, dextran fatty acid esters, sorbitol, sorbitol fatty acid esters, gelatin, serum albumins, and combinations thereof.

In the present invention, preferred surfactants or foaming agents are selected from the group consisting of phospholipids, nonionic surfactants, neutral or anionic surfactants, fluorinated surfactants, which can be neutral or anionic, and combinations of such emulsifying or foaming agents.

The nonionic surfactants suitable for use in the present invention include polyoxyethylene-polyoxypropylene copolymers. An example of such class of compounds is Pluronic, such as Pluronic F-68. Also contemplated are polyoxyethylene fatty acids esters, such as polyoxyethylene stearates, polyoxyethylene fatty alcohol ethers, polyoxyethylated sorbitan fatty acid esters, glycerol polyethylene glycol oxystearate, glycerol polyethylene glycol ricinoleate, ethoxylated soybean sterols, ethoxylated castor oils, and the hydrogenated derivatives thereof, and cholesterol. Anionic surfactants, particularly fatty acids (or their salts) having 12 to 24 carbon atoms, may also be used. One example of a suitable anionic surfactant is oleic acid, or its salt, sodium oleate.

It will be appreciated that a wide range of surfactants can be used. Indeed, virtually any surfactant or foaming agent (including those still to be developed) capable of facilitating formation of the microbubbles can be used in the present invention. The optimum surfactant or foaming agent or combination thereof for a given application can be determined through empirical studies that do not require undue experimentation. Consequently, one practicing the art of the present invention should choose the surfactant or foaming agents or combination thereof based upon such properties as biocompatibility or their non-Newtonian behavior.

The blood persistence of a contrast agent is inversely proportional to the LaPlace pressure which is proportional to the surface tension of the bubble. Reduced surface tension, therefore, increases blood persistence. Surfactants that form ordered structures (multilaminar sheets and rods) in solution and produce non-Newtonian viscoelastic surface tensions are especially useful. Such surfactants include many of the sugar based surfactants and protein or glycoprotein surfactants (including bovine, human, or other lung surfactants). One preferred type of such surfactant has a sugar or other carbohydrate head group, and a hydrocarbon or fluorocarbon tail group. A large number of sugars are known that can function as head groups, including glucose, sucrose, mannose, lactose, fructose, dextrose, aldose, and the like. The tail group preferably has from about 2 or 4 to 20 or 24 carbon atoms, and may be, for example, a fatty acid group (branched or unbranched, saturated or unsaturated) covalently bound to the sugar through an ester bond. The surface tension of bubbles produced with these surfactants greatly decreases as the surface is compressed by shrinkage of the bubble (e.g., when the bubble shrinks), and it is

increased as the surface area of the bubble is increased (e.g., when the bubble grows). This effect retards disproportionation, which leads to narrower size distribution and longer persisting bubbles in the vial and in vivo. A preferred surfactant mixture that has the properties associated with non-Newtonian viscoelasticity includes a nonionic surfactant or other foaming surfactant in combination with one of the non-Newtonian viscoelastic surfactant such as one of the sugar esters (e.g. 2% Pluronic F-68 plus 1% sucrose stearate). Often the ratio of the nonionic surfactant to the non-Newtonian surfactant is from about 5:1 to about 1:5, with the surfactants together (whether non-Newtonian or more conventional) comprising 0.5 to 8%, more preferably about 1 to 5% (w/v) of the microbubble-forming liquid mixture.

The lowering of surface tension in small bubbles, counter to typical LaPlace pressure, allows the use of more efficient gas osmotic agents such as higher molecular weight perfluorocarbons as the gas osmotic agent. With conventional surfactants, the higher molecular weight PFCs will condense at the high bubble pressures. Without these efficient surfactants higher boiling less membrane permeable PFCs, e.g. C_6F_{14}, would be extremely difficult.

One may also incorporate other agents within the aqueous phase. Such agents may advantageously include conventional viscosity modifiers, buffers such as phosphate buffers or other conventional biocompatible buffers or pH adjusting agents such as acids or bases, osmotic agents (to provide isotonicity, hyperosmolarity, or hyposmolarity). Preferred solutions have a pH of about 7 and are isotonic. However, when CO_2 is used as a first gas in a bubble designed to shrink rapidly to a first size, a basic pH can facilitate rapid shrinkage by removing CO_2 as it leaves the bubble, preventing a buildup of dissolved CO_2 in the aqueous phase.

B. The Gas Phase

A major aspect of the present invention is in the selection of the gas phase. As was discussed above, the invention relies on the use of combinations of gases to harness or cause differentials in partial pressures and to generate gas osmotic pressures, which stabilize the bubbles. The primary modifier gas is preferably air or a gas present in air. Air and/or fractions thereof are also present in normal blood and serum. Where the microbubbles are to be used in an environment different from blood, the primary modifier gas is preferably selected from gases normally present in the external medium. Another criteria is the ease with which the primary modifier gas is diffused into or out of the bubbles. Typically, air and/or fractions thereof are also readily permeable through conventional flexible or rigid bubble surfaces. These criteria, in combination, allow for the rapid diffusion of the primary modifier gas into or out of the bubbles, as required.

Modifier gases not present in the external medium can also be used. However, in this case the bubble will initially grow or shrink (depending on the relative permeability and concentrations of the external gases to the modifier) as the external gases replace the original modifier gas. If, during this process, the gas osmotic agent has not condensed, the bubble will remain stable.

The gas osmotic agent is preferably a gas that is less permeable through the bubble's surface than the modifier. It is also preferable that the gas osmotic agent is less soluble in blood and serum. Therefore, it will now be understood that the gas osmotic agent can be a gas at room or body temperature or it can ordinarily be a liquid at body temperature, so long as it has a sufficient partial or vapor pressure at the temperature of use to provide the desired osmotic effect.

Accordingly, fluorocarbons or other compounds that are not gases at room or body temperature can be used, provided that they have sufficient vapor pressure, preferably at least about 50 or 100 Torr at body temperature, or more preferably at least about 150 or 200 Torr. It should be noted that where the gas osmotic agent is a mixture of gases, the relevant measure of vapor pressure is the vapor pressure of the mixture, not necessarily the vapor pressure of the individual components of the mixed gas osmotic agent.

It is also important that where a perfluorocarbon is used as the osmotic agent within a bubble, the particular perfluorocarbon does not condense at the partial pressure present in the bubble and at body temperature. Depending on the relative concentrations of the primary modifier gas and the gas osmotic agent, the primary modifier gas may rapidly leave the bubble causing it to shrink and concentrate the secondary gas osmotic agent. Such shrinking may occur until the gas osmotic pressure equals the external pressure on the bubble (maximum absolute arterial pressure) plus the LaPlace pressure of the bubble minus the air tension, or air saturation tension, of the blood (essentially one atmosphere). Thus the condensing partial pressure of the resulting gas mixture at 37° C. must be above the equilibrium partial pressure, discussed above, of the osmotic agent. Representative fluorocarbons meeting these criteria and in increasing ability to stabilize microbubbles are as follows:

$$CCl_2F_2<CF_4,\ CHClF_2<C_4F_{10},\ N(C_2F_5)_3<C_5F_{12}<C_6F_{14}$$

Accordingly, it will be understood that PFC's with eight carbons atoms or fewer (37° C. vapor pressures greater than 80 mm Hg) are preferred. As will also be understood, however, it is possible to construct larger molecules with increased volatility through the addition of heteroatoms and the like. Therefore, the determination of the optimal secondary gas osmotic agent or gases agents is not size limited, but, rather, is based upon its ability to retain its vapor phase at body temperature and while providing a gas osmotic pressure equal to at least the sum of the arterial and LaPlace pressures.

A listing of some compounds possessing suitable solubility and vapor pressure criteria is provided in Table I:

TABLE I

perfluoro propanes, C_3F_8
perfluoro butanes, C_4F_{10}
perfluoro cyclo butanes, C_4F_8
perfluoro pentanes, C_5F_{12}
perfluoro cyclo pentanes, C_5F_{10}
perfluoro methylcyclobutanes, C_5F_{10}
perfluoro hexanes, C_6F_{14}
perfluoro cyclohexanes, C_6F_{12}
perfluoro methyl cyclopentanes, C_6F_{12}
perfluoro dimethyl cyclobutanes, C_6F_{12}
perfluoro heptanes, C_7F_{16}
perfluoro cycloheptanes, C_7F_{14}
perfluoro methyl cyclohexanes, C_7F_{14}
perfluoro dimethyl cyclopentanes, C_7F_{14}
perfluoro trimethyl cyclobutanes, C_7F_{14}
perfluoro triethylamines, $N(C_2F_5)_3$

It will be appreciated that one of ordinary skill in the art can readily determine other compounds that would perform suitably in the present invention that do not meet both the solubility and vapor pressure criteria, described above. Rather, it will be understood that certain compounds can be considered outside the preferred range in either solubility or vapor pressure, if such compounds compensate for the

aberration in the other category and provide a superior insolubility or low vapor pressure.

It should also be noted that for medical uses the gases, both the modifier gas and the gas osmotic agent, should be biocompatible or not be physiologically deleterious. Ultimately, the microbubbles containing the gas phase will decay and the gas phase will be released into the blood either as a dissolved gas or as submicron droplets of the condensed liquid. It will be understood that gases will primarily be removed from the body through lung respiration or through a combination of respiration and other metabolic pathways in the reticuloendothelial system.

Appropriate gas combinations of the primary modifier and secondary gases can be ascertained empirically without undue experimentation. Such empirical determinations are described in the Examples.

When an efficient surfactant, e.g., bovine lung surfactant, is employed to produce a large diameter bubble with a low surface tension, the LaPlace pressure is very low. When perfluorooctylbromide (PFOB) saturated air is inside the bubble and the bubble is exposed to air or a liquid nearly saturated with air (e.g., equilibrated with air) the gas osmotic pressure is greater than the LaPlace pressure and therefore the bubble grows. With smaller diameter bubbles the LaPlace pressure is higher and therefore the bubble shrinks and collapses. This shrinkage is at a reduced rate being driven by the difference between the LaPlace pressure minus reduced by the gas osmotic pressure. When small diameter bubbles are created by sonicating gas or gas vapor mixtures in a low surface tension surfactant solution, e.g., 2% pluronic F-68 plus 1% sucrose stearate, the time the bubbles persist in vitro, as observed by microscope, and in vivo as observed by Doppler ultrasound imaging of a rabbit's kidney post intravenous injection, correlated with the above gas osmotic pressure comparison.

In the rabbit kidney Doppler experiment (Example III), contrast enhancement was observed for up to 10 minutes with perfluorohexane/air mixtures in the bubbles compared with the instantaneous disappearance of contrast with pure air microbubbles. Thus, these perfluorochemicals are capable of exerting gas osmotic pressures that nearly counterbalance the LaPlace pressure and create functional ultrasound microbubble contrast agents.

A surprising discovery was that mixtures of PFCs, e.g., C_4F_{10} (as a combination modifier gas and a gas osmotic agent) saturated with C_6F_{14} vapor (as the main gas osmotic agent), can stabilize the bubble for longer times than either component alone. This is because C_4F_{10} is a gas at body temperature (and, thus, can act as both a modifier gas and a gas osmotic agent) has a somewhat reduced membrane permeability and it is only slightly soluble in C_6F_{14} at body temperature. In this situation the gas osmotic pressures of both agents are added together, leading to increased bubble persistence over that of air/C_6F_{14} only mixtures. It is possible that the condensing point of the longer persisting higher molecular weight C_6F_{14} component is increased, allowing a larger maximum gas osmotic pressure to be exerted. Other mixtures of PFCs will perform similarly. Preferred mixtures of PFCs will have ratios of 1:10 to 10:1, and include such mixtures as perfluorobutane/perfluorohexane and perfluorobutane/perfluoropentane. These preferred fluorochemicals can be branched or straight chain.

As was discussed above, we have also discovered that mixtures of nonosmotic gases in combination with the gas osmotic agent act to stabilize the size distribution of the bubbles before and after injection. Upon generation of the bubbles, the higher LaPlace pressures in smaller bubbles causes diffusion through the liquid phase to the lower LaPlace pressure larger bubbles. This causes the mean size distribution to increase above the capillary dimension limit of 5 microns with time. This is called disproportionation.

However, when a mixture of a modifier gases (e.g., air or carbon dioxide) are used with a gas osmotic agent (e.g., C_6F_{14}) a slight reduction in volume of the smaller bubbles, due to one of the modifier gases leaving the bubble, will concentrate the osmotic gas and increases its osmotic pressure, thus, retarding further shrinkage. On the other hand, the larger bubbles will increase in volume slightly, diluting the osmotic gas and also retarding further growth.

An additional advantage of using a mixture of an extremely blood soluble gas (e.g., 75% through 87.5% by volume CO_2) and an osmotic gas mixture (e.g. 28% C_6F_{14} vapor and 72% air) is that when injected, these bubbles rapidly shrink due to the loss of CO_2 to the blood. Carbon dioxide leaves particularly fast due to a specific plasma enzyme that catalyzes its dissolution. An 87.5% volume decrease due to loss of CO_2 corresponds with a halving of the bubble diameter. Accordingly, larger can be produced which will shrink to an appropriate size (i.e., 5 microns) upon injection or exposure to a solution with a basic or alkaline pH.

Accordingly, we have discovered that through use of a gas that is relatively hydrophobic and that has a relatively low membrane permeability, the rate of contrast particle decay can be reduced. Thus, through reducing the particle decay rate, the microbubbles' half lives are increased and contrast enhancement potential is extended.

II. Other Components.

It will be understood that other components can be included in the microbubble formulations of the present invention. For example, osmotic agents, stabilizers, chelators, buffers, viscosity modulators, air solubility modifiers, salts, and sugars can be added to fine tune the microbubble suspensions for maximum life and contrast enhancement effectiveness. Such considerations as sterility, isotonicity, and biocompatibility may govern the use of such conventional additives to injectable compositions. The use of such agents will be understood to those of ordinary skill in the art and the specific quantities, ratios, and types of agents can be determined empirically without undue experimentation.

III. Formation of the Microbubbles of the Present Invention.

There are a variety of methods to prepare microbubbles in accordance with the present invention. Sonication is preferred for the formation of microbubbles, i.e., through an ultrasound transmitting septum or by penetrating a septum with an ultrasound probe including an ultrasonically vibrating hypodermic needle. However, it will be appreciated that a variety of other techniques exist for bubble formation. For example, gas injection techniques can be used, such as venturi gas injection.

Other methods for forming microbubbles include formation of particulate microspheres through the ultrasonication of albumin or other protein as described in European Patent Application 0,359,246 by Molecular Biosystems, Inc.; the use of tensides and viscosity increasing agents as described in U.S. Pat. No. 4,446,442; lipid coated, non-liposomal, microbubbles as is described in U.S. Pat. No. 4,684,479; liposomes having entrapped gases as is described in U.S. Pat. Nos. 5,088,499 and 5,123,414; and the use of denatured albumin particulate microspheres as is described in U.S. Pat. No. 4,718,433. The disclosure of each of the foregoing patents and applications is hereby incorporated by reference.

Any of the above methods can be employed with similar success to entrain the modifier gases and gas osmotic agents of the present invention. Moreover, it is expected that similar enhancement in longevity of the bubbles created will be observed through use of the invention.

Sonication can be accomplished in a number of ways. For example, a vial containing a surfactant solution and gas in the headspace of the vial can be sonicated through a thin membrane. Preferably, the membrane is less than about 0.5 or 0.4 mm thick, and more preferably less than about 0.3 or even 0.2 mm thick, i.e., thinner than the wavelength of ultrasound in the material, in order to provide acceptable transmission and minimize membrane heating. The membrane can be made of materials such as rubber, Teflon, mylar, urethane, aluminized film, or any other sonically transparent synthetic or natural polymer film or film forming material. The sonication can be done by contacting or even depressing the membrane with an ultrasonic probe or with a focused ultrasound "beam." The ultrasonic probe can be disposable. In either event, the probe can be placed against or inserted through the membrane and into the liquid. Once the sonication is accomplished, the microbubble solution can be withdrawn from and vial and delivered to the patient.

Sonication can also be done within a syringe with a low power ultrasonically vibrated aspirating assembly on the syringe, similar to an inkjet printer. Also, a syringe or vial may be placed in and sonicated within a low power ultrasonic bath that focuses its energy at a point within the container.

Mechanical formation of microbubbles is also contemplated. For example, bubbles can be formed with a mechanical high shear valve (or double syringe needle) and two syringes, or an aspirator assembly on a syringe. Even simple shaking may be used. The shrinking bubble techniques described herein are particularly suitable for mechanically formed bubbles, having lower energy input than sonicated bubbles. Such bubbles will typically have a diameter much larger than the ultimately desired biocompatible imaging agent, but can be made to shrink to an appropriate size in accordance with the present invention.

In another method, microbubbles can be formed through the use of a liquid osmotic agent emulsion supersaturated with a modifier gas at elevated pressure introduced into in a surfactant solution. This production method works similarly to the opening of soda pop, where the gas foams upon release of pressure forming the bubbles.

In another method, bubbles can be formed similar to the foaming of shaving cream, where perfluorobutane, freon, or another like material that boils when pressure is released. However, in this method it is imperative that the emulsified liquid boils sufficiently low or that it contain numerous bubble nucleation sites so as to prevent superheating and supersaturation of the aqueous phase. This supersaturation will lead to the generation of a small number of large bubbles on a limited number of nucleation sites rather than the desired large number of small bubbles (one for each droplet).

In still another method, dry void-containing particles or other structures (such as hollow spheres or honeycombs) that rapidly dissolve or hydrate, preferably in an aqueous solution, e.g., albumin, microfine sugar crystals, hollow spray dried sugar, salts, hollow surfactant spheres, dried porous polymer spheres, dried porous hyaluronic acid, or substituted hyaluronic acid spheres, or even commercially available dried lactose microspheres can be stabilized with a gas osmotic agent.

For example, a spray dried surfactant solution can be formulated to obtain 5 micron or larger hollow spheres and packaged in a vial filled with an osmotic gas or a desired gas mixture as described herein. The gas will diffuse into the spheres. Diffusion can be aided by pressure or vacuum cycling. When reconstituted with a sterile solution the spheres will rapidly dissolve and leave osmotic gas stabilized bubbles in the vial. In the alternative, a lyophilized cake of surfactant and bulking reagents produced with a fine pore structure can be placed in a vial with a sterile solution and a head spaced with an osmotic gas mixture. The solution can be frozen rapidly to produce a fine ice crystal structure and, therefore, upon lyophilization produces fine pores (voids where the ice crystals were removed).

Alternatively, any dissolvable or soluble void-forming structures may be used. In this embodiment, where the void-forming material is not made from or does not contain surfactant, both surfactant and liquid are supplied into the container with the structures and the desired gas or gases. Upon reconstitution these voids trap the osmotic gas and, with the dissolution of the solid cake, form microbubbles with the gas or gases in them.

It will be appreciated that kits can be prepared for use in making the microbubble preparations of the present invention. These kits can include a container enclosing the gas or gases described above for forming the microbubbles, the liquid, and the surfactant. Alternatively, the container can contain the void forming material and the gas or gases, and the surfactant and liquid can be added to form the microbubbles. Alternatively, the surfactant can be present with the other materials in the container, and only the liquid needs to be added in order to form the microbubbles. Where a material necessary for forming the microbubbles is not already present in the container, it can be packaged with the other components of the kit, preferably in a form or container adapted to facilitate ready combination with the other components of the kit.

The container used in the kit may be of the type described elsewhere herein. In one embodiment, the container is a conventional septum-sealed vial. In another, it has a means for directing or permitting application of sufficient bubble forming energy into the contents of the container. This means can comprise, for example, the thin web or sheet described previously.

Any of the microbubble preparations of the present invention may be administered to a vertebrate, such as a bird or a mammal, as a contrast agent for ultrasonically imaging portions of the vertebrate. Preferably, the vertebrate is a human, and the portion that is imaged is the vasculature of the vertebrate. In this embodiment, a small quantity of microbubbles (e.g., 0.1 ml/Kg based on the body weight of the vertebrate) is introduced intravascularly into the animal. Other quantities of microbubbles, such as from about 0.005 ml/Kg to about 1.0 ml/Kg, can also be used. Imaging of the heart, arteries, veins, and organs rich in blood, such as liver, lungs, and kidneys can be ultrasonically imaged with this technique.

The foregoing description will be more fully understood with reference to the following Examples. Such Examples, are, however, exemplary of preferred methods of practicing the present invention and are not limiting of the scope of the invention or the claims appended hereto.

EXAMPLE I

Preparation of Microbubbles Through Sonication

Microbubbles with an average number weighted size of 5 microns were prepared by sonication of an isotonic aqueous phase containing 2% Pluronic F-68 and 1% sucrose stearate as surfactants, air as a modifier gas and perfluorohexane as the gas osmotic agent.

In this experiment, 1.3 ml of a sterile water solution containing 0.9% NaCl, 2% Pluronic F-68 and 1% sucrose stearate was added to a 2.0 ml vial. The vial had a remaining head space of 0.7 ml initially containing air. Air saturated with perfluorohexane vapor (220 torr of perfluorohexane with 540 torr of air) at 25 degrees C. was used to flush the headspace of the vial. The vial was sealed with a thin 0.22 mm polytetrafluoroethylene (PTFE) septum. The vial was turned horizontally, and a ⅛" (3 mm) sonication probe attached to a 50 watt sonicator model VC_{50}, available from Sonics & Materials was pressed gently against the septum. In this position, the septum separates the probe from the solution. Power was then applied to the probe and the solution was sonicated for 15 seconds, forming a white solution of finely divided microbubbles, having an average number weighted size of 5 microns as measured by Horiba LA-700 laser light scattering particle analyzer.

EXAMPLE II

Measurement of In-Vitro Size of Microbubbles

The in-vitro size of the microbubbles prepared in Example I was measured by laser light scattering. Studies of bubbles were conducted where the microbubbles were diluted into a 4% dextrose water solution (1:50) circulating through a Horiba LA-700 laser light scattering analyzer. The average microbubbles size was 5 microns and doubled in size in 25 minutes.

Interestingly, microbubbles prepared through the same method in Example I without the use of a gas osmotic agent (substituting air for the perfluorohexane/air mixture) had an average size of 11 microns and gave only background readings on the particle analyzer at 10 seconds.

EXAMPLE III

Measurement of In-Vivo Lifetime of Microbubbles

The lifetimes of microbubbles prepared in accordance with Example I were measured in rabbits through injecting 0.2 ml of freshly formed microbubbles into the marginal ear vein of a rabbit that was under observation with a Accuson 128XP/5 ultrasound imaging instrument with a 5 megahertz transducer. Several tests were conducted, during which images of the heart, inferior vena cava/aorta, and kidney were obtained while measuring the time and extent of the observable contrast enhancement. The results are presented in the following Table II:

TABLE II

ORGAN	DOSE	TIME MAX. INTENSITY	TIME TO MINIMUM USABLE INTENSITY	TIME TO NO ENHANCEMENT
Heart	0.1 ml/Kg	7–10 sec.	8–10 min.	25 min.
IVC/Aorta	0.1 ml/Kg	7–10 sec.	8–10 min.	25 min.
Kidney	0.1 ml/Kg	7–10 sec.	8–10 min.	25 min.

In Table III, a comparison of microbubbles prepared in an identical fashion without the use of an osmotic gas is presented (only air was used). Note that sporadic reflections were observed only in the right heart ventricle during the injection but disappeared immediately post dosing.

TABLE III

ORGAN	DOSE	TIME TO MAXIMUM INTENSITY	TIME TO MINIMUM USABLE INTENSITY	TIME TO NO ENHANCEMENT
Organ	0.1 ml/Kg	0	0	0
IVC/Aorta	0.1 ml/Kg	0	0	0
Kidney	0.1 ml/Kg	0	0	0

The use of an osmotic or gas osmotic agent dramatically increased the length of time for which microbubbles are visible.

EXAMPLE IV

Preparation of Mixed Osmotically Stabilized Microbubbles

Microbubbles with an average number weighted size of 5 microns were prepared by sonication of an isotonic aqueous phase containing 2% Pluronic F-68 and 1% sucrose stearate as surfactants and mixtures of perfluorohexane and perfluorobutane as the gas osmotic agents.

In this experiment, 1.3 ml of a sterile water solution containing 0.9% NaCl and 2% Pluronic F-68 was added to a 2.0 ml vial. The vial had a remaining head space of 0.7 ml, initially containing air. An osmotic gas mixture of perfluorohexane, 540 Torr and perfluorobutane at 220 Torr was used to flush the headspace before sealing with a thin 0.22 mm PTFE septum. The vial was sonicated as in Example I, forming a white solution of finely divided microbubbles, having an average particle size of 5 microns as measured by a Horiba LA-700 laser light scattering particle analyzer. This procedure was repeated twice more, once with pure perfluorobutane and then with a 540 Torr air+220 Torr perfluorohexane mixture. Vascular persistence of all three preparations was determined by ultrasound imaging of a rabbit post I.V. injection and are listed below

1.5 minutes	perfluorobutane
2 minutes	perfluorohexane + air
3 minutes	perfluorbutane + perfluorohexane

The mixture of perfluorocarbons persisted longer than either agent alone.

EXAMPLE V

Preparation of Gas Osmotically Stabilized Microbubbles from Soluble Spray Dried Spheres

Gas osmotically stabilized microbubbles were prepared by dissolving hollow spray dried lactose spheres, filled with an air perfluorohexane vapor mixture, in a surfactant solution.

Spray dried spheres of lactose with a mean diameter of approximately 100 micron and containing numerous 10 to 50 micron cavities, was obtained from DMV International under the trade name of Pharmatose DCL-11. Ninety milligrams of the lactose spheres was placed in a 2.0 ml vial. The porous spheres were filled with a mixture of 220 Torr perfluorohexane and 540 Torr air by cycling the gas pressure in the vial between one atmosphere and ½ atmosphere a total of 12 times over 5 minutes. A surfactant solution containing 0.9% sodium chloride, 2% Pluronic-F_{68} and 1% sucrose stearate was warmed to approximately 45° C., to speed the dissolution of the lactose, before injecting 1.5 ml of the warmed solution into the vial. The vial was then gently agitated by inversion for approximately 30 seconds to dissolve the lactose before injecting the microbubbles thus prepared into the Horiba LA-700 particle analyzer. A 7.7 micron volume weighted median diameter was measured approximately one minute after dissolution. The diameter of these microbubbles remained nearly constant, changing to a median diameter of 7.1 microns in 10 minutes. When the experiment was repeated with air filled lactose, the particle analyzer gave only background readings one minute after dissolution, thus demonstrating that gas osmotically stabilized microbubbles can be produced by the dissolution of gas-filled cavity-containing structures.

EXAMPLE VI

Preparation of Larger Bubbles that Shrink to a Desired Size

Microbubbles with an average volume weighted size of 20 microns shrinking to 2 microns were prepared by sonication of an isotonic aqueous phase containing 2% Pluronic F-68 as the surfactant, CO_2 as a diluent gas and perfluorohexane as the gas osmotic-agent.

In this experiment, 1.3 ml of a sterile water solution containing 0.9% NaCl, 2% Pluronic F-68 and 1% sucrose stearate was added to a 2.0 ml vial. The vial had a remaining head space of 0.7 ml initially containing air. A mixture of air saturated with perfluorohexane at 25 degrees C. diluted by a factor of 10 with CO_2 (684 Torr CO_2+54 Torr air+22 Torr perfluorohexane) was used to flush the head space. The vial was sealed with a thin 0.22 mm PTFE septum. The vial was sonicated as in Example I, forming a white solution of finely divided microbubbles, having an average particle size of 28 microns as measured by Horiba LA-700 laser light scattering analyzer. In the 4% dextrose+0.25 mM NaOH solution of the Horiba, the average bubble diameter rapidly shrank in 2 to 4 minutes from 28 microns to 5 to 7 microns, and then remained relatively constant, reaching 2.6 micron after 27 minutes. This is because the CO_2 leaves the microbubbles by dissolving into the water phase.

EXAMPLE VII

Perfluoroheptane Stabilized Microbubble In Vitro Experiment

Microbubbles were prepared as in Example I above employing perfluoroheptane saturated air (75 torr plus 685 torr air) and were measured as in Example II above. The average number weighted diameter of these microbubbles was 7.6 micron, one minute after circulation, and 2.2 microns after 8 minutes of circulation. This persistence, compared to the near immediate disappearance of microbubbles containing only air, demonstrates the gas osmotic stabilization of perfluoroheptane.

EXAMPLE VIII

Perfluorotripropyl Amine Stabilized Microbubble In Vivo Experiment

Microbubbles were prepared as in Example I above, employing perfluorotripropyl amine saturated air and were assessed as in Example III above. The usable vascular persistence of these microbubbles was found to be 2.5 minutes, thus demonstrating the gas osmotic stabilization of perfluorotripropyl amine.

EXAMPLE IX

Effect of a Non Newtonian Viscoelastic Surfactant—Sucrose Stearate

Microbubbles were prepared as in Example I above employing 0.9% NaCl, 2% Pluronic F-68 and 2% sucrose stearate as the surfactant and with perfluoropropane saturated air and perfluorohexane saturated air in the headspace. These two preparations were repeated with the same surfactant solution minus sucrose stearate. All four microbubble preparations were assessed as in Example III above. The usable vascular persistence of these microbubbles are listed below:

2% Pluronic F-68+2% sucrose stearate persistence
2 minutes perfluoropropane
4 minutes perfluorohexane
2% Pluronic F-68 only persistence
minutes perfluoropropane
minute perfluorohexane

As seen above, the reduced surface tension made possible by the non-Newtonian viscoelastic properties of sucrose stearate prevented the less volatile perfluorohexane from condensing, allowing perfluorohexane microbubbles of longer persistence to be produced.

elements of the kit may in some embodiments be the same as recited above for the microbubble preparation per se.

In another embodiment, the kit comprises a container, dried liquid-soluble void-containing structures in the container, the void-containing structures defining a plurality of voids having an average diameter less than about 100 μm, a gas in the voids, and a surfactant, wherein the void-containing structures, the gas, and the surfactant are together adapted to form microbubbles upon addition to the container of a liquid in which the void-containing structures are soluble. These void-containing structures can be made at least in part of the surfactant, e.g., by lyophilization of void-forming material or by spray drying, or can be formed from any other liquid soluble (preferably water soluble) film-forming material, such as albumin, enzymes, or other proteins, simple or complex carbohydrates or polysaccharides, and the like. The surfactants used in the kit can advantageously be those described above in connection with the microbubble preparations per se.

The present invention also includes a method for forming microbubbles, comprising the steps of providing a first gas, a second gas, a membrane forming material, and a liquid, wherein the first gas and the second gas are present in a molar ratio of from about 1:100 to about 1,000:1, and wherein the first gas has a vapor pressure of at least about (760–x) mm Hg at 37° C., where x is the vapor pressure of the second gas at 37° C., and wherein the vapor pressure of each of the first and second gases is greater than about 75 mm Hg at 37° C., with the proviso that the first gas and the second gas are not water vapor, and surrounding the first and second gases with the membrane forming material to form microbubbles in the liquid. The membrane forming materials and gases may be as described above. The method preferably further comprises the steps of initially forming microbubbles having a first average diameter wherein the initial ratio of the first gas to the second gas in the microbubbles is at least about 1:1, contacting the microbubbles having a first average diameter with a liquid medium, shrinking the microbubbles in the medium as a result of loss of the first gas through the membrane, and then stabilizing the microbubbles at a second average diameter of less than about 75% of the first diameter for a period of at least one minute. Preferably, the microbubbles are stabilized at the second diameter by providing a gas osmotic pressure differential across the membrane such that the tension of a gas or gases dissolved in the medium is greater than or equal to the pressure of the same gas or gases inside the microbubbles. In one embodiment, the first diameter is at least about 5 μm.

The invention also includes a method for forming microbubbles, comprising the steps of providing dried liquid-soluble void-containing structures, the void-containing structures defining a plurality of voids having a diameter less than about 100 μm, providing a gas in the voids, providing a surfactant, combining together the void-containing structures, the gas, the surfactant, and a liquid in which the void-containing structures are soluble, and dissolving the void-containing structures in the liquid whereby the gas in the enclosures forms microbubbles that are surrounded by the surfactant. As with the kit, preferred void-containing structures are formed of protein, surfactant, carbohydrate, or any of the other materials described above.

Finally, the present invention includes a method for imaging an object or body, comprising the steps of introducing into the object or body any of the aforementioned microbubble preparations and then ultrasonically imaging at least a portion of the object or body. Preferably, the body is a vertebrate and the preparation is introduced into the vasculature of the vertebrate. The method may further include preparing the microbubbles in any of the aforementioned manners prior to introduction into the animal.

DETAILED DESCRIPTION OF THE INVENTION

As used in the present description and claims, the terms "vapor" and "gas" are used interchangeably. Similarly, when referring to the tension of dissolved gas in a liquid, the more familiar term "pressure" may be used interchangeably with "tension." "Gas osmotic pressure" is more fully defined below, but in a simple approximation can be thought of as the difference between the partial pressure of a gas inside a microbubble and the pressure or tension of that gas (either in a gas phase or dissolved in a liquid phase) outside of the microbubble, when the microbubble membrane is permeable to the gas. More precisely, it relates to differences in gas diffusion rates across a membrane. The term "membrane" is used to refer to the material surrounding or defining a microbubble, whether it be a surfactant, another film forming liquid, or a film forming solid or semisolid. "Microbubbles" are considered to be bubbles having a diameter between about 0.5 and 300 μm, preferably having a diameter no more than about 200, 100, or 50 μm, and for intravascular use, preferably not more than about 10, 8, 7, 6, or 5 μm (measured as average number weighted diameter of the microbubble composition). When referring to a "gas," it will be understood that mixtures of gases together having the requisite property fall within the definition, except where the context otherwise requires. Thus, air may typically be considered a "gas" herein.

The present invention provides microbubbles that have a prolonged longevity in vivo that are suitable for use as ultrasound contrast enhancement agents. Typical ultrasound contrast enhancement agents exhibit contrast enhancement potential for only about one pass through the arterial system, or a few seconds to about a minute, and thus do not survive past the aorta in a patient following intravenous injection. In comparison, contrast agents prepared in accordance with the present invention continue to demonstrate contrast enhancements lives measured in multiple passes through the entire circulatory system of a patient following intravenous injection. Bubble lives of several minutes are easily demonstrated. Such lengthening of contrast enhancement potential during ultrasound is highly advantageous. In addition, the contrast enhancement agents of the invention provide superior imaging; for example, clear, vivid, and distinct images of blood flowing through the heart, lungs, and kidneys are achieved. Thus small, nontoxic doses can be administered in a peripheral vein and used to enhance images of the entire body.

While bubbles have been shown to be the most efficient ultrasound scatterers for use in intravenous ultrasound contrast agents, their main practical drawback is the extremely short lifetime of the small (typically less than 5 microns diameter) bubbles required to pass through capillaries in suspension. This short lifetime is caused by the increased gas pressure inside the bubble, which results from the surface tension forces acting on the bubble. This elevated internal pressure increases as the diameter of the bubble is reduced. The increased internal gas pressure forces the gas inside the bubble to dissolve, resulting in bubble collapse as the gas is forced into solution. The LaPlace equation, $\Delta P=2\gamma/r$, (where ΔP is the increased gas pressure inside the bubble, γ is the surface tension of the bubble film, and r is the radius of the bubble) describes the pressure exerted on a

gas bubble by the surrounding bubble surface or film. The La Place pressure is inversely proportional to the bubble radius; thus, as the bubble shrinks, the La Place pressure increases, increasing the rate of diffusion of gas out of the bubble and the rate of bubble shrinkage.

It was surprisingly discovered that gases and gas vapor mixtures which can exert a gas osmotic pressure opposing the LaPlace pressure can greatly retard the collapse of these small diameter bubbles. In general, the invention uses a primary modifier gas or mixture of gases that dilute a gas osmotic agent to a partial pressure less than the gas osmotic agent's vapor pressure until the modifier gas will exchange with gases normally present in the external medium. The gas osmotic agent or agents are generally relatively hydrophobic and relatively bubble membrane impermeable and also further possess the ability to develop gas osmotic pressures greater than 75 or 100 Torr at a relatively low vapor pressure.

The process of the invention is related to the well known osmotic effect observed in a dialysis bag containing a solute that is substantially membrane impermeable (e.g. PEG, albumin, polysaccharide, starch) dissolved in an aqueous solution is exposed to a pure water external phase. The solute inside the bag dilutes the water inside the bag and thus reduces the rate of water diffusion out of the bag relative to the rate of pure water (full concentration) diffusion into the bag. The bag will expand in volume until an equilibrium is established with an elevated internal pressure within the bag which increases the outward diffusional flux rate of water to balance the inward flux rate of the pure water. This pressure difference is the osmotic pressure between the solutions.

In the above system, the internal pressure will slowly drop as the solute slowly diffuses out of the bag, thus reducing the internal solute concentration. Other materials dissolved in the solution surrounding the bag will reduce this pressure further, and, if they are more effective or at a higher concentration, will shrink the bag.

It was observed that bubbles of air saturated with selected perfluorocarbons grow rather than shrink when exposed to air dissolved in a liquid due to the gas osmotic pressure exerted by the perfluorocarbon vapor. The perfluorocarbon vapor is relatively impermeable to the bubble film and thus remains inside the bubble. The air inside the bubble is diluted by the perfluorocarbon, which acts to slow the air diffusion flux out of the bubble. This gas osmotic pressure is proportional to the concentration gradient of the perfluorocarbon vapor across the bubble film, the concentration of air surrounding the bubble, and the ratio of the bubble film permeability to air and to perfluorocarbon.

As discussed above, the LaPlace pressure is inversely proportional to the bubble radius; thus, as the bubble shrinks, the LaPlace pressure increases, increasing the rate of diffusion of gas out of the bubble and the rate of bubble shrinkage, and in some cases leading to the condensation and virtual disappearance of a gas in the bubble as the combined LaPlace and external pressures concentrate the osmotic agent until its partial pressure reaches the vapor pressure of liquid osmotic agent.

We have discovered that conventional microbubbles that contain any single gas will subsist in the blood for a length of time that depends primarily on the arterial pressure, the bubble diameter, the membrane permeability of the gas through the bubble's surface, the mechanical strength of the bubble's surface, the presence, absence, and concentration of the gases that are ordinarily present in the blood or serum, and the surface tension present at the surface of the bubble (which is primarily dependent on the diameter of the bubble and secondarily dependent on the identity and concentration of the surfactants which form the bubble's surface). Each of these parameters are interrelated, and they interact in the bubble to determine the length of time that the bubble will last in the blood.

The present invention includes the discovery that a single gas or a combination of gases can together act to stabilize the structure of the microbubbles entraining or entrapping them. Essentially, the invention utilizes a first gas or gases (a "primary modifier gas") that optionally is ordinarily present in normal blood and serum in combination with one or more additional second gases (a "gas osmotic agent or agents" or a "secondary gas") that act to regulate the osmotic pressure within the bubble. Through regulating the osmotic pressure of the bubble, the gas osmotic agent (defined herein as a single or mixture of chemical entities) exerts pressure within the bubble, aiding in preventing deflation. Optionally, the modifier gas may be a gas that is not ordinarily present in blood or serum. However, the modifier gas must be capable of diluting and maintaining the gas osmotic agent or agents at a partial pressure below the vapor pressure of the gas osmotic agent or agents while the gases in blood or other surrounding liquid diffuse into the bubble. In an aqueous medium, water vapor is not considered to be one of the "gases" in question. Similarly, when microbubbles are in a nonaqueous liquid medium, the vapor of that medium is not considered to be one of the "gases."

We have discovered that by adding a gas osmotic agent that has, for example, a reduced membrane permeability through the bubble's surface or reduced solubility in the external continuous phase liquid phase, the life of a bubble formed therewith will be radically increased. This stabilizing influence can be understood more readily through a discussion of certain theoretical bubbles. First, we will consider the effects of arterial pressure and surface tension on a hypothetical microbubble containing only air.

Initially, a hypothetical bubble containing only air is prepared. For purposes of discussion, this bubble will initially be considered to have no LaPlace pressure. Generally, when equilibrated at standard temperature and pressure (STP), it will have a internal pressure of 760 Torr of air and the surrounding fluid air concentration will also be equilibrated at 760 Torr (i.e., the fluid has an air tension of 760 Torr). Such a bubble will neither shrink nor grow.

Next, when the above hypothetical bubble is introduced into the arterial system, the partial pressure of air (or air tension) in the blood (the air pressure at which the blood was saturated with air) will also be approximately 760 Torr and there will be an arterial pressure (for the purposes of the this discussion at 100 Torr). This total creates an external pressure on the bubble of 860 Torr, and causing the gases in the bubble to be compressed until the internal pressure increases to 860 Torr. There then arises a difference of 100 Torr between the air pressure inside the bubble and the air tension of the fluid surrounding the bubble. This pressure differential causes air to diffuse out of the bubble, through its air-permeable surface membrane, causing the bubble to shrink (i.e., lose air) as it strives to reach equilibrium. The bubble shrinks until it disappears.

Next, consider the additional, and more realistic, effect on the hypothetical bubble of adding the surface tension of the bubble. The surface tension of the bubble leads to a LaPlace pressure exerted on gas inside the bubble. The total pressure exerted on the gas inside the bubble is computed through adding the sum of the atmospheric pressure, the arterial

pressure and the LaPlace pressure. In a 3 μm bubble a surface tension of 10 dynes per centimeter is attainable with well chosen surfactants. Thus, the LaPlace pressure exerted on the hypothetical 3 μm bubble is approximately 100 Torr and, in addition, the arterial pressure of 100 Torr is also exerted on the bubble. Therefore, in our hypothetical bubble, the total external pressure applied to the gas inside the bubble is 960 Torr.

The bubble will be compressed until the pressure of the air inside the bubble rises to 960 Torr. Accordingly, a concentration differential of 200 Torr arises between the air inside the bubble and the air dissolved in the blood. Therefore, the bubble will rapidly shrink and disappear even more rapidly than it did in the previous case, as it attempts to reach equilibrium.

The discovery of the present invention is illustrated by considering a third hypothetical microbubble containing air and a gas osmotic agent or a secondary gas. Assume that a theoretical bubble, initially having no arterial pressure and no LaPlace pressure, is prepared having a total pressure of 760 Torr, which is made up of air at a partial pressure of 684 Torr and a perfluorocarbon ("PFC") as a gas osmotic agent at a partial pressure of 76 Torr. Further, assume that the perfluorocarbon is selected to have one or more traits that make it capable of acting as an appropriate gas osmotic agent, such as limited bubble membrane permeability or limited solubility in the external liquid phase. There is an initial gas osmotic pressure differential between the 684 Torr of air within the bubble and the 760 Torr of air tension outside the bubble (assuming STP) of 76 Torr. This 76 Torr initial pressure difference is the initial gas osmotic pressure and will cause the bubble to expand. Air from outside of the bubble will diffuse into and inflate the bubble, driven by the osmotic pressure differential, similar to the way water diffuses into a dialysis bag containing a starch solution, and inflates the bag.

The maximum gas osmotic pressure this gas mixture can develop is related to the partial pressure of the PFC and the ratio of the permeability of the PFC to the permeability of the air in the surrounding fluid. In theory, and as observed experimentally, the bubble will grow indefinitely as the system attempts to reach osmotic equilibrium between the concentration of air (equivalent to the partial pressure of air) within the bubble and the concentration of air surrounding the bubble (the air tension).

When the hypothetical mixed gas bubble is exposed to 100 Torr of arterial pressure where the blood has a dissolved air tension of 760 Torr, the total external pressure will equal 860 Torr (760 Torr atmospheric pressure and 100 Torr arterial pressure). The bubble will compress under the arterial pressure, causing the internal pressure of the bubble to reach 860 Torr. The partial pressure of the air will increase to 774 Torr and the partial pressure of the PFC (the second gas) will increase to 86 Torr. The air will diffuse out of the bubble until it reaches osmotic equilibrium with the air dissolved in the blood (i.e., 760 Torr) and the partial pressure of the PFC will increase to 100 Torr. The partial pressure of the PFC will act to counterbalance the pressure exerted due to the arterial pressure, halting shrinkage of the bubble, in each case, assuming that the permeability of the bubble to the PFC is negligible.

When the surface tension or LaPlace pressure component of 100 Torr is added (as discussed above with the air bubble), a total of 200 Torr additional pressure is exerted on the gas in the bubble. Again, the bubble will compress until and the pressure inside the bubble increases to 960 Torr (partial pressure of air 864 and partial pressure of PFC 96). The air will diffuse from the bubble until it reaches 760 Torr (in equilibrium with the concentration of air the dissolved in the blood) and the partial pressure of the PFC will increase to 200 Torr, where, again, the gas osmotic pressure induced by the PFC will act to counterbalance the pressure exerted by the LaPlace pressure and the arterial pressure, again, assuming that the membrane permeability of the bubble to the PFC is negligible.

Similarly, if the partial pressure of air in the bubble is lower than the air tension in the surrounding liquid, the bubble will actually grow until the PFC is sufficiently diluted by incoming air so that the pressure of air inside and the air tension outside of the bubble are identical.

Thus, it can be seen has been shown that bubbles can be effectively stabilized through the use of combinations of gases, since the correct combination of gases will result in a gas osmotic pressure differential that can be harnessed to counterbalance the effects of the LaPlace pressure and the arterial pressure exerted on the a gas within the bubble in circulating blood.

Examples of particular uses of the microbubbles of the present invention include perfusion imaging of the venous drainage system of the heart, the myocardial tissue, and determination of perfusion characteristics of the heart and its tissues during stress or exercise tests, or perfusion defects or changes due to myocardial infarction. Similarly, myocardial tissue can be viewed after oral or venous administration of drugs designed to increase the blood flow to a tissue. Also, visualization of changes in myocardial tissue due to or during various interventions, such as coronary tissue vein grafting, coronary angioplasty, or use of thrombolytic agents (TPA or streptokinase) can also be enhanced. As these contrast agents can be administered conveniently via a peripheral vein to enhance the visualization of the entire circulatory system, they will also aid in the diagnosis of Deep Vein Thrombosis and in the ability to ultrasonically monitor the fetus and the umbilical cord.

It should, however, be emphasized that these principles have application beyond ultrasound imaging. Indeed, the present invention is sufficiently broad to encompass the use of gas osmotic pressure to stabilize bubbles for uses in any systems, including nonbiological applications.

In a preferred embodiment, the microbubbles of the present invention have a surfactant-based bubble membrane. However, the principles of the invention can be applied to stabilize microbubbles of virtually any type. Thus, mixed gases or vapors of the type described above can stabilize albumin based bubbles, polysaccharide based microbubbles, spray dried microsphere derived microbubbles, and the like. This result is achieved through the entrapment, within the chosen microbubble, of a combination of gases, preferably a primary modifier gas or mixture of gases that will dilute a gas osmotic agent to a partial pressure less than the gas osmotic agent's vapor pressure until the modifier gas will exchange with gases normally present in the external medium. The gas osmotic agent or agents are generally relatively hydrophobic and relatively bubble membrane impermeable and also further possess the ability to develop gas osmotic pressures greater than 50, 75, or 100 Torr. In one preferred embodiment, the gas vapor pressure of the gas osmotic agent is preferably less than about 760 Torr at 37° C., preferably less than about 750, 740, 730, 720, 710, or 700 Torr, and in some embodiments less than about 650, 600, 500, or 400 Torr.

In preferred embodiments, the vapor pressure of the primary modifier gas is at least 660 Torr at 37° C. and the

The foregoing description details certain preferred embodiments of the present invention and describes the best mode contemplated. It will be appreciated, however, that no matter how detailed the foregoing appears in text, the invention can be practiced in many ways and the invention should be construed in accordance with the appended Claims and any equivalents thereof.

What is claimed is:

1. A microbubble preparation, comprising:

an aqueous medium containing a plurality of microbubbles, said microbubbles comprising:

a generally spherical microbubble membrane;

a first gas and a second component contained within said membrane, wherein said second component comprises the vapor of a compound that is a liquid at 37° C. and 760 mm Hg but which has a vapor pressure of at least 75 mm Hg at 37° C., wherein the first gas and the second component are respectively present in a molar ratio of about 1:100 to about 1000:1, with the proviso that said first gas and said second component are not water vapor.

2. The preparation of claim **1**, wherein said second component comprises a fluorocarbon and said first gas is a nonfluorocarbon.

3. The preparation of claim **2**, wherein said first gas comprises nitrogen, oxygen, carbon dioxide, or a mixture thereof.

4. The preparation of claim **2**, wherein:

said microbubbles are in a liquid medium and have a first average diameter;

the ratio of said first gas to said second component in said microbubbles is at least 1:1; and

said microbubbles are adapted to shrink in said medium as a result of loss of said first gas through said membrane to a second average diameter of less than about 75% of said first diameter and then remain stabilized at or about said second diameter for at least about 1 minute as a result of a gas osmotic pressure differential across said membrane.

5. The preparation of claim **4**, wherein said liquid medium contains gas or gases dissolved therein with a gas tension of at least about 700 mm Hg, wherein said first diameter is at least about 5 μm, and wherein the tension of the gas or gases dissolved in said medium is less than the pressure of the same gas or gases inside said microbubbles.

6. The preparation of claim **4**, wherein said first diameter is at least about 10 μm and said second diameter is between about 1 μm and 6 μm.

7. The preparation of claim **1**, wherein said second component has an average molecular weight at least about 4 times that of said first gas.

8. The preparation of claim **1**, wherein said molar ratio of said first gas to said second component is from about 1:10 to about 500:1.

9. The preparation of claim **1**, wherein said second component comprises a fluorocarbon and said first gas is a nonfluorocarbon.

10. The preparation of claim **9**, wherein said second component comprises at least two fluorocarbons.

11. The preparation of claim **9**, wherein said second component comprises a perfluorocarbon.

12. The preparation of claim **1**, wherein both said first gas and said second component comprise fluorocarbons.

13. The preparation of claim **1**, wherein said second component has a water solubility of not more than about 0.5 mM at 25° C. and one atmosphere, and wherein said first gas has a water solubility at least about 10 times greater than that of said second component.

14. The preparation of claim **1**, wherein the permeability of the membrane to said first gas is at least about 5 times greater than the permeability of said membrane to said second component.

15. The preparation of claim **1**, further comprising:

a liquid in said container in admixture with said microbubbles, wherein said container further comprises means for transmission of sufficient ultrasonic energy to said liquid to permit formation of said microbubbles by sonication.

16. The preparation of claim **15**, wherein said means for transmission comprises a flexible polymer material having a thickness less than about 0.5 mm.

17. The preparation of claim **1**, wherein said membrane is a surfactant.

18. The preparation of claim **17**, wherein said surfactant comprises a non-Newtonian viscoelastic surfactant.

19. The preparation of claim **17**, wherein said surfactant is a carbohydrate.

20. The preparation of claim **19**, wherein said carbohydrate is a polysaccharide.

21. The preparation of claim **17**, wherein said surfactant is a fatty acid ester of a sugar.

22. The preparation of claim **17**, wherein said surfactant is sucrose stearate.

23. The preparation of claim **17**, wherein said surfactant is a phospholipid.

24. The preparation of claim **1**, wherein said membrane is solid or semi-solid.

25. The preparation of claim **1**, wherein said membrane is a proteinaceous material.

26. The preparation of claim **25**, wherein said proteinaceous material is albumin.

27. The preparation of claim **2**, wherein:

said microbubbles have a first average diameter;

the ratio of said first gas to said second component in said microbubbles is at least 1:1; and

said microbubbles are adapted to shrink in blood in vivo as a result of loss of said first gas through said membrane to a second average diameter of less than about 75% of said first diameter and then remain stabilized at or about said second diameter for at least about 1 minute as a result of a gas osmotic pressure differential across said membrane.

28. The preparation of claim **27**, wherein said first diameter is at least about 5 μm, and wherein the tension of the gas or gases dissolved in said blood is less than the pressure of the same gas or gases inside said microbubbles.

29. The preparation of claim **27**, wherein said first diameter is at least about 10 μm and said second diameter is between about 1 μm and 6 μm.

30. The preparation of claim **27**, wherein said second component has an average molecular weight at least about 4 times that of said first gas.

31. The preparation of claim **27**, wherein said molar ratio of said first gas to said second component is from about 1:10 to about 500:1.

32. The preparation of claim **27**, wherein said second component comprises a fluorocarbon and said first gas is a nonfluorocarbon.

33. The preparation of claim **32**, wherein said second component comprises at least two fluorocarbons.

34. The preparation of claim **32**, wherein said second component comprises a perfluorocarbon.

35. The preparation of claim **27** wherein both said first gas and said second component comprise fluorocarbons.

36. The preparation of claim 1, wherein said microbubbles contain as said first gas, or as said second component, or respectively as said first gas and said second component, gaseous perfluorobutane and perfluorohexane in a ratio from about 1:10 to about 10:1.

37. The preparation of claim 1, wherein said microbubbles contain as said first gas, or as said second component, or respectively as said first gas and said second component, gaseous perfluorobutane and perfluoropentane in a ratio from about 1:10-to-about 10:1.

38. The preparation of claim 4, wherein said medium is aqueous.

39. A kit for use in preparing microbubbles, comprising:

a sealed container;

a liquid in said container;

a surfactant in said container; and

a fluorocarbon gas in said container, wherein said liquid, said surfactant, and said fluorocarbon gas are together adapted to form microbubbles upon the application of energy to the sealed container.

40. The kit of claim 39, further comprising

means in said container for permitting transmission of sufficient external ultrasonic energy to said liquid to form microbubbles in said container.

41. The kit of claim 40, wherein said means for transmission comprises a flexible polymer membrane having a thickness less than about 0.5 mm.

42. The kit of claim 39, further comprising:

a nonfluorocarbon gas in said container, wherein the molar ratio of said nonfluorocarbon gas to said fluorocarbon gas is from about 1:10 to about 1000:1, with the proviso that said nonfluorocarbon gas is not water vapor.

43. A kit for use in preparing microbubbles, comprising:

a container;

dried liquid-soluble void-containing structures in said container, said void-containing structures defining voids having an average diameter less than about 100 μm;

a liquid suitable for in vivo injection in which said void-containing structures are substantially soluble;

a gas in said voids, wherein said gas is the vapor of a non-aqueous material that is a liquid at 37° C.; and

a surfactant, wherein said void-containing structures, said gas, and said surfactant are together adapted to form microbubbles upon addition to said container of said liquid.

44. The kit of claim 43, wherein said void-containing structures comprise at least in part said surfactant.

45. The kit of claim 43, wherein said surfactant is a non-Newtonian viscoelastic surfactant.

46. The kit of claim 43, wherein said surfactant is a fatty acid ester of a sugar.

47. The kit of claim 43, wherein said surfactant is a phospholipid.

48. The kit of claim 43, wherein said void-containing structures are proteinaceous.

49. The kit of claim 43, wherein said void-containing structures are formed of a carbohydrate.

50. The kit of claim 43, wherein said gas is a fluorocarbon.

51. The kit of claim 43, wherein said gas comprises perfluorohexane.

52. The kit of claim 43, wherein said void-containing structures comprise spray dried microspheres.

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[54] **GAS-FILLED MICROSPHERES WITH FLUORINE-CONTAINING SHELLS**

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[52] **U.S. Cl.** **424/9.52**; 424/9.5

[58] **Field of Search** 424/9.52, 9.51, 424/9.5; 252/312, 351

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[57] **ABSTRACT**

Improved ultrasonic imaging contrast agents are provided which are comprised of an aqueous suspension of microspheres comprising at least one gas, preferably a perfluorocarbon, encapsulated by elastic shells of a biocompatible, fluorine-containing amphiphilic material, processes for making the microspheres, and methods of diagnostic imaging using the improved contrast agents.

19 Claims, No Drawings

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GAS-FILLED MICROSPHERES WITH FLUORINE-CONTAINING SHELLS

DESCRIPTION

1. Technical Field

This invention is in the field of ultrasonic imaging. More particularly it relates to microspheres useful for ultrasonic imaging which comprise microbubbles of gas encapsulated by shells composed of a biocompatible, fluorine-containing amphiphilic material, aqueous suspensions of such microspheres and the use of such suspensions in ultrasonic imaging.

2. Background

Diagnostic ultrasonic imaging is based on the principle that waves of sound energy can be focused upon an area of interest and reflected in such a way as to produce an image thereof. The ultrasonic transducer is placed on a body surface overlying the area to be imaged, and ultrasonic energy in the form of sound waves is directed toward that area. As ultrasonic energy travels through the body, the velocity of the energy and acoustic properties of the body tissue and substances encountered by the energy determine the degree of absorption, scattering, transmission and reflection of the ultrasonic energy. The transducer then detects the amount and characteristics of the reflected ultrasonic energy and translates the data into images.

As ultrasound waves move through one substance to another there is some degree of reflection at the interface. The degree of reflection is related to the acoustic properties of the substances defining the interface. If these acoustic properties differ, such as with liquid-solid or liquid-gas interfaces, the degree of reflection is enhanced. For this reason, gas-containing contrast agents are particularly efficient at reflecting ultrasound waves. Thus, such contrast agents intensify the degree of reflectivity of substances encountered and enhance the definition of ultrasonic images.

Ophir and Parker describe two types of gas-containing imaging agents: (1) free gas bubbles; and (2) encapsulated gas bubbles (*Ultrasound in Medicine and Biology* 15(4):319–333 (1989)), the latter having been developed in an attempt to overcome instability and toxicity problems encountered using the former. Encapsulated gas bubbles, hereinafter referred to as "microspheres", are composed of a microbubble of gas surrounded by a shell of protein or other biocompatible material. One such imaging agent is ALBUNEX® (Molecular Biosystems, Inc., San Diego, Calif.) which consists of a suspension of air-filled albumin microspheres.

Generally, microspheres of a particular gas exhibit improved in vivo stability when compared to free bubbles of the same gas. However, most microspheres still have relatively short in vivo half lives which compromise their usefulness as contrast agents. This instability in vivo was thought to result from the collapse or breakdown of the shells under pressure resulting in rapid diffusion of the gas from the microspheres. Thus, many recent efforts have centered on improvements to the shell as a way of increasing in vivo stability. Known improvements relating to protein-shelled microspheres include coating the protein shell with surfactants (Giddy, WO 92/05806) and chemical cross-linking of the protein shell (Holmes et al., WO92/17213).

Additional efforts directed toward improving microsphere stability include the use of non-proteinaceous shell-forming materials. Bichon et al. (EPA 458,745 A1) and Schneider et al. (*Inv. Radiol.* 27:134–139 (1992)) describe the production

of polymeric "microballoons" made of interfacially deposited polymers encapsulating various gases such as carbon dioxide, nitrous oxide, methane, freon, helium and other rare gases. Klaveness (WO92/17212) describe the use of chemically-linked, non-proteinaceous amphiphilic moieties encapsulating "air, nitrogen, oxygen, hydrogen, nitrous oxide, carbon dioxide, helium, argon, sulfur hexafluoride and low molecular weight, optionally fluorinated, hydrocarbons such as methane, acetylene or carbon tetrafluoride." Erbel et al. (U.S. Pat. No. 5,190,982) describe the use of polyamino-dicarboxylic acid-co-imide derivatives.

More recently, Schneider, et al. (European Patent Application 554,213 A1) have demonstrated that microspheres containing gases with certain physical properties have improved stability. It is theorized that microsphere instability is caused by the increase in pressure to which microspheres are exposed once they are introduced into the circulatory system. Although Schneider, et al. do not speculate as to the mechanism responsible for their observed enhanced pressure resistance, we believe it is due to the effects of gas solubility on the rate of gas exchange with the aqueous environment.

According to the principles of Henry's law, as pressure increases, the solubility of a given gas in solution will also increase. When a bubble of gas in solution is subjected to pressure, the rate of gas exchange between the gas in the bubble and the surrounding solution will increase in proportion to the amount of pressure, and the bubble of gas will eventually become completely solubilized. The more insoluble the gas is in the surrounding solution, the longer it will take for a bubble to become completely solubilized.

If the bubble of gas is surrounded by a shell, i.e in the form of a microsphere, the effects of gas exchange are still observed, since microsphere shells do not completely eliminate the contact between the gas in the microsphere and the surrounding solution. Hence, when microspheres suspended in solution are subjected to pressure, the gas inside the microspheres eventually becomes solubilized in the surrounding solution which results in collapse of the microspheres.

Microspheres useful for ultrasonic imaging typically have shells with a certain degree of elasticity. This property is necessary for two important reasons. Firstly, microspheres having shells which are rigid may resonate at frequencies higher than those used for ultrasonic imaging which lessens their efficiency as contrast enhancers. Secondly, rigid-shelled microspheres can crack or break when subjected to pressure thus releasing their gaseous contents into the aqueous environment. Elastic-shelled microspheres while able to overcome the aforementioned problems may unfortunately be more susceptible to the effects of gas exchange with the aqueous environment because of their tendency to be more permeable. This results in a greater degree of contact between the gas inside the microsphere and the surrounding aqueous environment thus facilitating gas exchange.

In order to inhibit the exchange of gas in the microsphere center with the surrounding aqueous environment, the present invention describes the introduction of fluorine into the microsphere shell material. Microspheres having fluorine-containing shells will exhibit decreased water permeability and thus enhanced resistance to pressure instability due to gas exchange.

DISCLOSURE OF THE INVENTION

The present invention provides compositions and methods of ultrasonic imaging using novel gas-filled micro-

spheres that have fluorine-containing shells. In particular, the present invention provides compositions for use as ultrasonic imaging agents comprising aqueous suspension of microspheres, the microspheres comprising a fluorine-containing shell formed from amphiphilic, biocompatible material surrounding a microbubble of at least one biocompatible gas.

The gas is preferably insoluble and is more preferably fluorinated and even more preferably a C_1 to C_5 perfluorocarbon. Suitable perfluorinated gases include perfluoromethane, perfluoroethane, perfluoropropane, perfluorobutane and perfluoropentane.

Suitable fluorine-containing shell material includes lipids, proteins (which includes both naturally occurring proteins and synthetic amino acid polymers), synthetic organic polymers and mixtures and copolymers thereof. The shell material is preferably a protein, and more preferably human serum albumin.

The present invention also provides a process for making microspheres with fluorine-containing shells which involves reacting the shell material with a fluorine-containing reactive compound which effects the introduction of fluorine moieties into the shell material.

The present invention further provides a method to enhance the contrast of tissues and organs in an ultrasonic image comprising the steps of injecting the above described composition into a subject and detecting an ultrasonic image.

MODES OF CARRYING OUT THE INVENTION

The present invention relates to stabilized microspheres which comprise a fluorine-containing shell formed from biocompatible material surrounding a microbubble of gas. Such shell material is less water permeable than its non-fluorine-containing equivalent. In addition, interactions which take place between certain gases and the shell may further stabilize the microsphere. In particular, when the gas also contains fluorine, the fluorine-fluorine interactions between the gas and shell provide an additional barrier to gas exchange with the surrounding aqueous environment.

Suitable shell material must be amphiphilic, i.e., containing both hydrophobic and hydrophilic moieties. It must also be capable of forming a thin layer or skin around the encapsulated gas, which will generally result in hydrophilic groups oriented externally and hydrophobic groups oriented internally. When microspheres are produced to contain insoluble gas, this orientation is believed to be enhanced by the presence of the insoluble gas during microsphere formation.

The shell thus formed must also be solid. The term solid is used to refer to the state of matter at the temperature of a subject being imaged which is distinguished from either the liquid or gaseous state, and is characterized generally as being discrete, non-fluid and capable of maintaining form or shape. Compositions which are quasi-liquid at the temperature at which the subject is imaged (the imaging temperature), such as certain lipids having transition temperatures close (i.e. within 15° C.) to imaging (body) temperature have some of the characteristics of both liquids and solids. These quasi-liquids are also contemplated by the present invention and included in the term solid. The thickness of a microsphere shell will depend primarily on its rigidity when formed but will generally be in the range of 10 to 500 nm.

Different classes of materials that would be suitable for forming microsphere shells include, but are not limited to, lipids, proteins (both naturally occurring and synthetic amino acid polymers), synthetic organic polymers, and mixtures or copolymers thereof. Lipid shells can be formed from either naturally occurring or synthetic lipids, for example, phospholipids, such as phosphoglycerides, phosphatidic acid, phosphatidylcholine, phosphatidyl serine, phosphatidylethanolamine, phosphatidyl inositol, phosphatidylglycerol, diphosphatidyl-glycerol (cardiolipin); glycolipids, such as cerebrosides, galactocerebrosides, glucocerebrosides, sphingomyelin, sphingolipids, derivatized with mono-, di- and trihexosides, sulfatides, glycosphingolipid, and lysophosphatidylcholine; unsaturated fatty acids, such as palmitoleic acid, oleic aid, vaccenic acid, linoleic acid, α-linolenic acid and arachidonic acid; saturated fatty acids, such as myristic acid, palmitic acid, stearic acid, arachidic acid, behenic acid, lignoceric acid and cerotic acid; mono-, di- and triglycerides; and steroids, such as cholesterol, cholesterol esters, cholestanol, ergosterol, coprostanol, squalene, and lanosterol.

Shells consisting predominantly of lipids will generally be oriented with the hydrophobic side adjacent the gas while the hydrophilic side forms the external microsphere surface. The hydrophilic moieties of most lipids are polar, i.e., cationic or anionic such as the phosphate moiety of a phospholipid, or they can be zwitterionic as in phosphatidyl cholines. Alternatively, lipids without polar groups can be made polar such as by introduction of non-ionic hydrophilic moieties, for example polyethylene glycol, or carbohydrates.

Phospholipids are a particularly useful sub-class of lipid shell materials. The various phospholipids have characteristic phase transition temperatures, Tc, below which the fatty acyl chains form a quasi-crystalline structure and above which the chains are in a more quasi-liquid state. Their ability to transition from quasi-crystalline to quasi-liquid with increases in temperature can facilitate the production of microspheres that become more elastic in-vivo. For example, using a phospholipid with a Tc which is between 25° C. and 37° C., a solid shelled microsphere can be formed at room temperature (20°–25° C.) which becomes less rigid at an imaging temperature of 37° C. This may lead to enhanced echogenicity due to improved shell elasticity. Phospholipids having lower Tc values, for example, dimyristoyl or dipentadecanoyl glycerophosphocholine, are particularly suitable for use in this aspect of the invention.

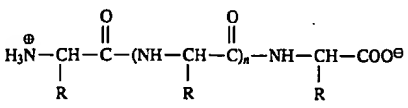
A comparison of the Tc values of a series of synthetic L-α-lecithins (1,2-diacyl-sn glycero-3-phosphocholines, or glycerophosphocholines) reveals that Tc increases steadily relative to hydrocarbon chain length. Dipalmitoyl-glycerophosphocholine has a Tc of 41° C., while the dimyristoyl derivative has a Tc of 23° C. The disteroyl and diarachidoyl derivatives have Tcs of 55° C. and 66° C., respectively. It is also contemplated that a mixture of these and other phospholipids that have different Tc values could also be used to achieve the desired transitional characteristics of the microsphere shells. Further, the gas in the microsphere and the introduction of fluorine into the shell material may alter the Tc value. This effect should be considered when selecting the phospholipid.

Lipid shells may also optionally incorporate proteins, amino acid polymers, carbohydrates or other substances useful for altering the rigidity, elasticity, biodegradability and/or biodistribution characteristics of the shell. Incorporation of sterols is particularly useful in increasing the rigidity of the shell. The rigidity of the shell can also be enhanced by cross-linking, for example, with irradiation.

Protein shell material includes both naturally-occurring proteins and synthetic amino acid polymers which herein are

both generally referred to as being in the class of shell materials described as "proteins". Examples of naturally-occurring proteins include gamma-globulin (human), apotransferrin (human), beta-lactoglobulin, urease, lysozyme, and albumin. Synthetic amino acid polymers can optionally be in the form of block or random co-polymers combining both hydrophobic and hydrophilic amino acids in the same or different chains.

The structure of a protein or an amino acid polymer is represented as:



wherein R is the side chain of the amino acid (for example, the R of cysteine is $HSCH_2$) The amino acid side chain will also generally be the fluorine-containing portion of the protein/polymer.

Synthetic organic polymers are also suitable for forming microsphere shells. These polymers can consist of a single repeating unit or different repeating units which form a random, alternating or block-type co-polymer. These organic polymers include cross-linked polyelectrolytes such as phosphazenes, imino-substituted polyphosphazenes, polyacrylic acids, polymethacrylic acids, polyvinyl acetates, polyvinyl amines, polyvinyl pyridine, polyvinyl imidazole, and ionic salts thereof. Cross-linking of these polyelectrolytes is accomplished by reaction with multivalent ions of the opposite charge. Further stabilization can be accomplished by adding a polymer of the same charge as the polyelectrolyte. See U.S. Pat. No. 5,149,543 which is incorporated herein by reference.

Additional synthetic organic monomeric repeating units which can be used to form polymers suitable for shell materials within the present invention are hydroxyacids, lactones, lactides, glycolides, acryl containing compounds, aminotriazol, orthoesters, anhydrides, ester imides, imides, acetals, urethanes, vinyl alcohols, enolketones, and organosiloxanes.

The introduction of fluorine into the shell material can be accomplished by any known method. For example, the introduction of perfluoro-t-butyl moieties is described in U.S. Pat. No. 5,234,680; SYNTHESIS OF FLUOROORGANIC COMPOUNDS (Springer-Verlag, New York, 1985); Zeifman, Y. V. et al., Uspekhi Khimii (1984) 53 p. 431; and Dyatkin, B. L. et al., Uspekhi Khimii (1976) 45, p. 1205. These methods generally involve the reaction of perfluoroalkyl carbanions with host molecules as follows:

$$(CF_3)_3C^- + R{-}X > (CF_3)_3C{-}R$$

where R is a host molecule and X is a good leaving group, such as Br, Cl, I or a sulfonato group. After adding a leaving group to the foregoing monomeric shell materials using methods well known in the art, perfluoro-t-butyl moieties can then be easily introduced to these derivatized shell materials (the host molecules) in the manner described above.

Additional methods are known for the introduction of trifluoromethyl groups into various organic compounds. One such method describes the introduction of trifluoromethyl groups by nucleophilic perfluoroalkylation using perfluoroalkyl-trialkylsilanes. (SYNTHETIC FLUORINE CHEMISTRY pp. 227–245 (John Wiley & Sons, Inc., New York, 1992)).

Fluorine can be introduced into any of the aforementioned shell materials either in their monomeric or polymeric form. Preferably, fluorine moieties are introduced into monomers, such as fatty acids, amino acids or polymerizable synthetic organic compounds, which are then polymerized for subsequent use as microsphere shell-forming material.

The introduction of fluorine into the shell material may also be accomplished by forming microspheres in the presence of a perfluorocarbon gas. For example, when microspheres are formed from proteins such as human serum albumin in the presence of a perfluorocarbon gas, such as perfluoropropane, using mechanical cavitation, fluorine from the gas phase becomes bound to the protein shell during formation. The presence of fluorine in the shell material can be later detected by NMR of shell debris which has been purified from disrupted microspheres. Fluorine can also be introduced into microsphere shell material using other methods for forming microspheres, such as sonication, spray-drying or emulsification techniques.

Another way in which fluorine can be introduced into the shell material is by using a fluorine-containing reactive compound. The term "reactive compound" refers to compounds which are capable of interacting with the shell material in such a manner that fluorine moieties become covalently attached to the shell material. When the shell forming material is a protein, preferred reactive compounds are either alkyl esters or acyl halides which are capable of reacting with the protein's amino groups to form an amide linkage via an acylation reaction (see ADVANCED ORGANIC CHEMISTRY pp. 417–418 (John Wiley & Sons, New York, N.Y., 4th ed., 1992)). The reactive compound can be introduced at any stage during microsphere formation, but is preferably added to the gas phase prior to microsphere formation. For example, when microspheres are to be made using mechanical or ultrasound cavitation techniques, the reactive compound can be added to the gas phase by bubbling the gas to be used in the formation of the microspheres (starting gas) through a solution of the reactive compound. This solution is kept at a constant temperature which is sufficient to introduce a desired amount of reactive compound into the gas phase. The resultant gas mixture, which now contains the starting gas and the reactive compound, is then used to form microspheres. The microspheres are preferably formed by sonication of human serum albumin in the presence of the gas mixture as described in U.S. Pat. No. 4,957,656, which is incorporated herein by reference.

Suitable fluorine-containing alkyl esters and acyl halides are provided in Table I:

TABLE I

REACTIVE COMPOUND	BOILING POINT* (°C.)
ALKYL ESTERS:	
diethyl hexafluoroglutarate	75 (at 3 mm Hg)
diethyl tetrafluorosuccinate	78 (at 5 mm Hg)
methyl heptafluorobutyrate	95
ethyl heptafluorobutyrate	80
ethyl pentafluoropropionate	76
methyl pentafluoropropionate	60
ethyl perfluorooctanoate	167
methyl perfluorooctanoate	159
ACYL HALIDES:	
nonafluoropentanoyl chloride	70
perfluoropropionyl chloride	8
hexafluoroglutaryl chloride	111
heptafluorobutyryl chloride	38

*at 1 atm (760 mm Hg) unless otherwise noted above

In addition to the use of alkyl esters and acid halides described above, it is well known to those skilled in synthetic organic chemistry that many other fluorine-containing reactive compounds can be synthesized, such as aldehydes, isocyanates, isothiocyanates, epoxides, sulfonyl halides,

anhydrides, acid halides and alkyl sulfonates, which contain perfluorocarbon moieties (—CF_3, —C_2F_5, —C_3F_4, —$C(CF_3)_3$). These reactive compounds can then be used to introduce fluorine moieties into any of the aforementioned shell materials by choosing a combination which is appropriate to achieve covalent attachment of the fluorine moiety.

Sufficient fluorine should be introduced to decrease the permeability of the microsphere shell to the aqueous environment. This will result in a slower rate of gas exchange with the aqueous environment which is evidenced by enhanced pressure resistance. Although the specific amount of fluorine necessary to stabilize the microsphere will depend on the shell material and the gas contained therein, after introduction of fluorine the shell material will preferably contain 0.5 to 20 percent by weight, and more preferably 1 to 10 percent by weight.

Gases suitable for use within the present invention are pharmacologically acceptable, i.e., biocompatible and minimally toxic to humans. The term "biocompatible" means the ability of the gas to be metabolized without the formation of toxic by-products. The term "gas" refers to any compound which is a gas or capable of forming gas at the temperature at which imaging is being performed (typically normal physiological temperature). The gas may be composed of a single compound or a mixture of compounds. Examples of gases suitable for use within the present invention are air, O_2, N_2, H_2, CO_2, N_2O; noble gases such as argon, helium, xenon; hydrocarbon gases such as methane, ethane, propane, n-butane, isobutane and pentane, and perfluorocarbon gases such as perfluoromethane, perfluoroethane, perfluoropropane, perfluorobutane, perfluoroisobutane and perfluoropentane. The gas is preferably a perfluorocarbon which is insoluble in water, which intends a solubility of less than 0.01 mL of gas per mL of water at atmospheric pressure and a temperature of 25° C. This degree of insolubility results in maximum stability in vitro and persistence in vivo. Solubility can be determined by any appropriate method. See, for example, Wen-Yang Wen et al. (1979) *J. Solubility Chem.* 8(3):225–246. A non-exhaustive list of preferred insoluble gases suitable for use within the present invention is provided in Table II.

different from those of blood or tissue. The maximum size (mean diameter) of the microsphere is defined by that size which will pass through the pulmonary capillaries. In the case of humans, that size will typically be less than about 10 micrometers. Correspondingly, the minimum size is that which will provide efficient acoustic scattering at the ultrasonic frequencies typically used for ultrasonic imaging. (The frequency may vary with the mode of imaging, e.g., transthoracic, transesophageal, and will normally be in the range of 2–12 MHz.) The minimum size will typically be about 0.1 micrometers. The typical mean size of the microspheres used in the invention method will be about 2 to about 7 micrometers. This size will permit their passage through capillaries, if necessary, without being filtered out prior to reaching the area to be imaged (e.g., where a peripheral venous injection site is used). Thus, microspheres within the present invention will be capable of perfusing tissue and producing an enhanced image of the tissue, organs and any differentiation between well-perfused and poorly-perfused tissue, without being injected into the arteries or directly into the area to be imaged. Accordingly, they may be injected into a peripheral vein or other predetermined area of the body, resulting in considerably less invasion than the arterial injections required for an angiogram.

Microspheres within the present invention may be used for imaging a wide variety of areas. These areas include, but are not limited to, myocardial tissue, liver, spleen, kidney, and other tissues and organs presently imaged by ultrasonic techniques. Use of microspheres within the present invention may result in an enhancement of such currently obtainable images.

Suspensions of microspheres are made by diluting the microspheres after formation to the desired concentration preferably 5×10^7 to 5×10^9 microspheres per mL, of suspending liquid which can be any aqueous, biologically-compatible liquid. Examples of such liquids are buffers, saline, protein solutions and sugar solutions.

A microsphere suspension within the present invention is stable both in vivo and in vitro. Stability in vivo is a function of the ability of a concentrated suspension (approximately 1×10^9 microspheres per mL) to withstand 40 pounds per

TABLE II

FORMULA	NAME	MOLECULAR WEIGHT (g/mol)	BOILING POINT (°C.)	WATER SOLUBILITY at 25° C. and 1 atm (mL/mL × 10^{-3})
SF_6	sulfur hexafluoride	146	−64	5.40
CF_4	perfluoromethane	88	−130	5.04
C_2F_6	perfluoroethane	138	−78	1.38
$CF_3CF_2CF_3$	perfluoropropane	188	−37	<1
$CF_3(CF_2)_2CF_3$	perfluorobutane	238	−2	<1
$CF_3(CF_2)_3CF_3$	perfluoropentane	288	29.5	<1

The microspheres of the present invention may be made by known methods used to make conventional gas-filled microspheres such as sonication, mechanical cavitation using a milling apparatus, or emulsion techniques. Such techniques are exemplified in U.S. Pat. Nos. 4,957,656; 5,137,928; 5,190,982; 5,149,543: PCT Application Nos. WO 92/17212; WO 92/18164; WO 91/09629; WO 89/06978; WO 92/17213; GB 91/12823; and WO 93/02712: and EPA Nos. 458,745 and 534,213 which are incorporated herein by reference.

The microspheres of the present invention are echogenic (i.e., capable of reflecting sound waves) being composed of material having acoustic properties which are significantly

square inch (psi) pressure as evidenced by no appreciable change in size distribution after one minute at this pressure.

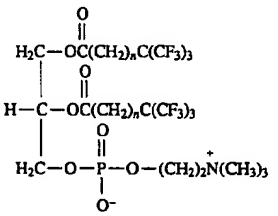
In terms of method of operation, the use of the subject microspheres would be the same as that of conventional ultrasonic contrast agents. The amount of microspheres used would be dependent on a number of factors including the choice of liquid carriers (water, sugar solution, etc.), degree of opacity desired, areas of the body to be imaged, site of injection and number of injections. In all instances, however, sufficient microspheres would be used in the liquid carrier to achieve enhancement of discernable images by the use of ultrasonic scanning.

The invention is further illustrated by the following examples. These examples are not intended to limit the invention in any manner.

EXAMPLE 1

Preparation of Microsphere Consisting of a Lipid-Based Material Encapsulating an Insoluble Gas

A phosphatidyl choline is fluorinated as follows: A ω-bromo carboxylic acid ester ($Br(CH_2)_nCOOCH_2CH_3$) and perfluorisobutylene ($(CF_3)_2CF{=}CF_2$) are reacted in the presence of CsF and monoglyme at room temperature to form a fluorinated ester ($(CF_3)_3C(CH_2)_nCOOCH_2CH_3$). This ester is hydrolyzed to form a free acid ($(CF_3)_3C(CH_2)_nCOOH$) which is converted to the acylchloride ($(CF_3)_3C(CH_2)_nCOCl$) by reacting it with thionyl chloride. The acylchloride is reacted in the presence of base with glycerophosphocholine to form the fluorinated glycerophosphocholine as follows:



The length of the carbon chain of the bromo carboxylic acid ester used can be varied, for example between C5 and C20.

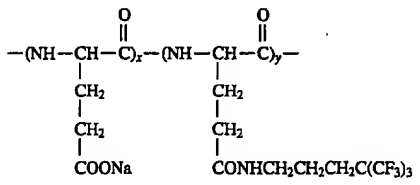
Microspheres are formed by first emulsifying the following ingredients to form an oil-in-water emulsion: fluorinated glycerophosphocholine (either alone or in combination with other lecithins), an insoluble gas (for example, see Table II above) and water. Optionally, the emulsion contains triolein, cholesterol and/or α-tocopherol. Homogenization of the emulsion is carried out under pressure and at a temperature above the transition temperature of the fluorinated glycerophosphocholine, followed by cooling to room temperature.

EXAMPLE 2

Preparation of a Synthetic Amino Acid Polymer Containing Fluorine Using a Polymer as the Starting Material

A polyglutamic acid polymer containing fluorine (polysodium L-glutamate-co-perfluoro-t-butyl propylglutamine) was prepared as follows: Poly L-glutamic acid (m.w. 95,000, 1.77 g, 13.7 mmol) was dissolved in 40 mL of dimethylformamide (DMF) at 50° C. After cooling to room temperature, 10 mL pyridine, 1-hydroxybenzotriazole (1.85 g, 13.7 mmol) and perfluoro-t-butyl-propylamine hydrochloride (2.15 g, 6.85 mmol) were added. The reaction mixture was rendered anhydrous by evaporation of pyridine in vacuo. Dicyclohexylcarbodiimide (2.82 g, 13.7 mmol) was added and the solution stirred at room temperature for 48 hours. N,N'-dicyclohexylurea was removed by filtration and the filtrate poured into water adjusted to pH 3.0. The precipitate formed was filtered off and subsequently dissolved in water at pH 8.0. Undissolved material was removed by filtration (0.22μ membrane filter). The polymer solution was dialyzed overnight to remove soluble low-molecular weight material. The polymer solution was lyophilized yielding a white sponge-like material consisting of poly sodium L-glutamate-co-perfluoro-t-butyl propylglutamine.

The resultant fluorinated polyglutamic acid has the structure:



with the fluorinated moieties being present randomly in approximately 40–50% of the glutamic acid residues in the polymer.

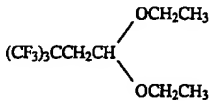
The polymer is then added to human serum albumin, for example in a ratio of 1:10, and microspheres are produced as described in Examples 4 or 5.

EXAMPLE 3

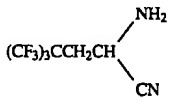
Preparation of a Synthetic Amino Acid Polymer Containing Fluorine Using a Monomer as the Starting Material

A poly-amino acid polymer containing fluorine (poly-3-(perfluoro-t-butyl)-2-aminobutyric acid) is synthesized as follows:

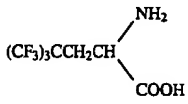
Bromoacetaldehyde diethyl acetal is reacted with perfluoroisobutylene in the presence of CsF and diglyme to yield:



Acid hydrolysis of the diethyl acetal gives the aldehyde. Strecker synthesis with ammonium cyanide yields the corresponding amino nitrile:



Hydrolysis gives the following amino acid derivative:



This compound is polymerized either alone or with other amino acids using known methods to form a fluorine-containing synthetic amino acid polymer.

The polymer is then added to human serum albumin, for example in a ratio of 1:10, and microspheres are produced as described in Examples 4 or 5.

EXAMPLE 4

Method of Making Microspheres by Mechanical Cavitation

Microspheres are produced using the shell-forming materials of Example 2 or 3 as follows: A 5% solution is deaerated under continuous vacuum for two hours. The

vacuum is released by filling the evacuated vessel with the gas to be used for formation of the microspheres. The solution is adjusted to a temperature (about 68° C.) necessary to achieve local denaturation of the albumin upon cavitation via an in line heat exchanger and pumped at about 100 mL/min into a colloid mill, for example, a 2" colloid mill (Greerco, Hudson N.H., model W250V or AF Gaulin, Everett, Mass., model 2F). The gas, at room temperature, is added to the liquid feed just upstream of the inlet port at a flow rate of about 120–220 mL/min. The gap between the rotor and the stator is adjusted to about 3/1000th inch and the albumin solution is milled continuously at about 7000 rpm at a process temperature of about 73° C.

The dense white solution of microspheres thus formed is immediately chilled to a temperature of about 10° C. by a heat exchanger, and collected in glass vials. The vials are immediately sealed.

EXAMPLE 5

Method of Making Microspheres by Sonic Cavitation

Microspheres are produced using the shell-forming materials of Example 2 or 3 as follows: A 5% solution is deaerated under continuous vacuum for two hours. The vacuum is released by filling the evacuated vessel with the gas to be used for formation of the microspheres. The continuous sonication process is performed as described by Cerny (U.S. Pat. No. 4,957,656).

The dense white solution of microspheres thus formed is immediately chilled to a temperature of about 10° C. by a heat exchanger, and collected in glass vials. The vials are immediately sealed.

EXAMPLE 6

Pressure Resistance of Microspheres

Microspheres with fluorine-containing shells are prepared as described in Examples 4 or 5 above. A ten mL aliquot of each suspension adjusted to a concentration of approximately 1×10^9 microspheres per mL in phosphate buffered saline is placed in a 10 mL glass gas-tight syringe (Hamilton, Reno Nev.) fitted with a pressure gauge. All headspace is removed and the apparatus is sealed. A constant pressure of about 40 psi is applied for about 3 minutes. A Coulter Counter is used to measure the sample particle concentration and distribution. Stable microspheres exhibit no significant change (less than 10%) in the mean size of the microspheres after application of pressure.

EXAMPLE 7

Elasticity

Microspheres with fluorine-containing shells are prepared as described in Examples 4 or 5 above. Microspheres are diluted into phosphate buffered saline to a concentration of approximately 1×10^9 microspheres per mL and placed in a clear cell positioned on the stage of a microscope. The cell is connected to a nitrogen source that allows observation of the effects of rapid application and release of up to 3 psi pressure on the microspheres. Elastic microspheres are capable of returning to their original dimensions after release of applied pressure.

EXAMPLE 8

Diagnostic Imaging

Microspheres prepared as described in Examples 4 and 5 are used in diagnostic imaging as follows: For a dog weighing approximately 25 Kg, a 1.0 mL volume of a microsphere suspension containing 5×10^7 to 5×10^9 microspheres per mL is injected into a peripheral (cephalic) vein at a rate of 0.3 mL per second. Images of the heart are acquired using a Hewlett Packard Sonos 1500 (Andover, Mass.) ultrasonograph in the B-mode using a transthoracic 5.0 mHz transducer. Images are recorded at a frame rate of 30 frames per second throughout the procedure and stored on S-VHS tape for later processing.

What is claimed is:

1. A composition for use as an ultrasonic imaging agent comprising an aqueous suspension of microspheres, said microspheres comprising a shell of fluorine-containing amphiphilic, biocompatible material surrounding a microbubble of at least one biocompatible gas.

2. The composition of claim **1**, wherein the gas has a solubility of less than 0.01 mL per mL of water at 25° C. and 1 atm.

3. The composition of claim **2**, wherein the gas is a perfluorocarbon.

4. The composition of claim **3**, wherein the perfluorocarbon gas is selected from the group consisting of perfluoromethane, perfluoroethane, perfluoropropane, perfluorobutane and perfluoropentane.

5. The composition of claim **1**, wherein the gas is a hydrocarbon.

6. The composition of claim **5**, wherein the hydrocarbon gas is selected from the group consisting of methane, ethane, propane, n-butane, isobutane and pentane.

7. The composition of claim **1**, wherein the shell comprises a fluorine-containing amphiphilic, biocompatible material selected from the group consisting of lipids, proteins, and synthetic organic polymers.

8. The composition of claim **1**, wherein the fluorine-containing amphiphilic biocompatible material is a lipid.

9. The composition of claim **8**, wherein the lipid is a phospholipid.

10. The composition of claim **1**, wherein the fluorine-containing amphiphilic biocompatible material is a protein.

11. The composition of claim **10** wherein the protein is human serum albumin.

12. The composition of claim **1**, wherein the fluorine-containing amphiphilic biocompatible material is a synthetic organic polymer.

13. The composition of claim **1** wherein the microsphere shells contain 0.5 to 20 percent by weight fluorine.

14. A process for making pressure resistant microspheres, said microspheres comprising a shell of fluorine-containing amphiphilic, biocompatible material surrounding a microbubble of at least one biocompatible gas, said process comprising the steps of:

(a) introducing fluorine into an amphiphilic, biocompatible material by reacting a fluorine-containing reactive compound with said amphiphilic, biocompatible material to form a fluorine-containing amphiphilic, biocompatible shell material; and

(b) simultaneously or subsequently forming said microspheres from said fluorine-containing amphiphilic, biocompatible shell material and said biocompatible gas.

15. The process of claim **14** wherein the reactive compound is selected from the group consisting of aldehydes,

isocyanates, isothiocyanates, epoxides, alkyl esters, acyl halides, sulfonyl halides, anhydrides, acid halides, and alkyl sulfonates.

16. The process of claim **14** wherein the reactive compound contains at least one perfluorocarbon moiety selected from the group consisting of $-CF_3$, $-C_2F_5$, $-C_3F_7$ and $-C(CF_3)_3$.

17. The process of claim **14**, further comprising forming the microspheres in the presence of at least one gas having a solubility of less than 0.01 mL per mL of water at 25° C. and 1 atm.

18. The process of claim **17** wherein the gas is a perfluorocarbon gas.

19. A method to enhance the contrast of tissues and organs of a patient in an ultrasonic image comprising:

(a) injecting the composition of claim **1** into the patient; and

(b) ultrasonically imaging the tissues and organs while the composition is present therein.

* * * * *